How individual olfactory receptors affect olfactory guided behavior in *Drosophila*

Dissertation

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by Dipl. Biol. Tom Retzke

born on July 14th, 1988 in Wolfen, Germany

Reviewers

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Date of public defense

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"Stay awhile and listen!"
(Deckard Cain)

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Introduction

Up to date there is no general definition for what is life. But most of the definitions share some ideas. According to the Oxford Dictionary life "*includes the capacity of growth, reproduction, functional activity and continual change preceding death*". Koshland (2002) postulated seven pillars (program, improvisation, compartmentalization, energy, regeneration, adaptability, seclusion), which build the fundament of the temple of life. The common ground of those descriptions of life is the ability to move and to react to environmental changes. To fulfill the latter, living organisms are equipped with abilities to detect heat, sonic waves, light, wind, magnetic fields, etc. – commonly known as senses. One of the oldest among them is chemosensation. Although even unicellular organisms are able to detect changing concentrations of chemicals in water (Bondoc et al., 2016), higher animals, have developed two distinct chemical senses: gustation for the detection of close-range chemical cues, and olfaction for the detection of volatiles over sometimes amazingly long distances. One of the most famous model organisms to study olfaction is the fruit fly *Drosophila melanogaster*.

Drosophila melanogaster as a model organism

The first description of the vinegar fly *Drosophila melanogaster* was made 1830 by Johann Wilhelm Meigen, a German entomologist. 75 years later, in 1905, the first scientific paper dealing with *Drosophila* as a laboratory animal was published by Frederick W. Carpenter (Carpenter, 1905). Thomas Hunt Morgan, an American

geneticist, finally established the vinegar fly in the beginning of the 20th century in his laboratory (Davenport, 1941). Hence he paved the way to the success of this fly as one of the main biological models by investigating and revealing the general chromosomal structure and its meaning in heredity (Nobel Prize 1933). Furthermore the popularity of *Drosophila* as a model organism increased because of its short generation time of 8 to 13 days (Mavor, 1927), depending on temperature, and its simplicity to breed. Over decades and across disciplines this model organism supported several Nobel-Prize-awarded discoveries (Hermann Müller in 1946; Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus in 1995; Richard Axel and Linda Buck in 2004; Jules Hoffman, Bruce Beutler and Ralph Steinmann in 2011).

Investigations of the circadian rhythm (Konopka and Benzer, 1971) and the learning behavior (Dudai et al., 1976) of *Drosophila* form the fundament for the analysis of genetic basics in fly behavior. Since this time a huge amount of genetic tools were developed, allowing cell-specific manipulation, including the artificial activation or silencing and visualization of certain cell types or whole cell populations (Venken et al., 2011). Especially the olfactory system of the vinegar fly was spotlighted during the last decades using these approaches (de Bruyne et al., 2001; Couto et al., 2005; Vosshall and Stocker, 2007; Stensmyr et al., 2012; Grabe et al., 2014; Dweck et al, 2015).

The olfactory system of *Drosophila melanogaster*

Drosophila uses odors to detect, localize, and judge food (Stensmyr et al., 2012), oviposition sites (Dweck et al., 2013) and mating partners (Ziegler et al., 2013). Odors are emitted by different sources in a fly´s environment and are basically blends of many different molecules. To detect these molecules flies are equipped with two specialized paired head appendices, the antennae and the maxillary palps. Both olfactory organs are covered with hair like structures, so-called sensilla (Couto et al., 2005; Benton et al., 2009). While the antennae carry around 500 sensilla each, only 50 can be found on the maxillary palps (Grabe et al., 2016). At the end of the 20th century de Bruyne et al. (1999) showed that these sensilla have numerous pores on their surface. Odor molecules can travel through these pores and reach the sensillum lymph, where they are picked up by odor binding proteins (OBPs). OBPs serve as a kind of ferry to transport the – usually hydrophobic – odor molecules through the hydrous sensillum lymph to chemical receptors (Fan et al., 2011). Up to date we know three different classes of chemical receptors in *Drosophila*: olfactory receptors (ORs; Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), ionotropic glutamate receptors (IRs; Benton et al., 2009) and gustatory receptors (GRs; Clyne et al., 2000; Smith, 2001; Jones et al., 2007). At least for ORs and IRs specific co-receptors have been identified, which form a functional dimer with the chemoreceptors. 2004 Larsson et al. found that OR83b is ubiquitously expressed and they called it *orco* (olfactory receptor co-receptor) and Wicher et al. (2008) found evidence, that *orco* is necessary for signal transduction. While on the antennae we find ORs and IRs and two GRs detecting CO_2 (GR21a/GR63a; Jones et al., 2007; Kwon et al., 2007), the

maxillary palps carry ORs and IRs only and the remaining GRs are located on the flies´ labellum, legs, wings and the ovipositor (Stocker, 1994; Vosshall and Stocker, 2007; Liman et al., 2014).

On the antenna each sensillum hosts the OR or IR exposing dendrites of 1 – 4 olfactory sensory neurons (OSNs), which express – with a few exceptions – only one type of OR or IR (Fig. 1A; Couto et al., 2005). When odor molecules bind to the ligand-specific receptors the neuron becomes activated, indicated by an action potential. The generated signal spreads from the dendrites over the soma to the axon of an OSN. All OSN axons of one antenna are bunched and draft from the periphery to the protocerebrum, more precise to the antennal lobe (AL). The AL consists of spherical subunits, so-called glomeruli, and each glomerulus is innervated by all axons from OSNs expressing the same type of OR (Fig. 1B; Couto et al., 2005; Fishilevich and Vosshall, 2005). A glomerulus is basically a dense neuronal network where OSNs simply synapse on projection neurons (PNs), and form further connections *via* a third type of neurons, local interneurons (LNs), to both OSNs and PNs (Stocker et al., 1990; Stocker, 1994; Vosshall and Stocker, 2007; Chou et al., 2010; Seki et al., 2010). Further Liu and Wilson (2013) showed that glutamatergic LNs inhibit also GABAergic LNs, which broadens the range and flexibility of the olfactory system. The main role of LNs is thought to work as gain control (Wilson, 2013; Galizia, 2014). While excitatory LNs (eLNs) amplify a weak signal coming from OSNs, inhibitory LNs (iLNs) weaken a strong incoming signal (Olsen and Wilson, 2008; Kazama and Wilson, 2008; Root et al., 2008). However, 2013 Zhu et al. showed evidence in the adult zebrafish that there are also bimodal interneurons which inhibit mitral cells through GABAergic synapses and activate the same cells *via* gap junctions. This mechanisms guarantee a similar PN

activity pattern across a range of odor concentrations (Olsen et al., 2010), which is important for the fly to identify an odor in its natural environment (Murlis et al., 1992) independent from its concentration variability.

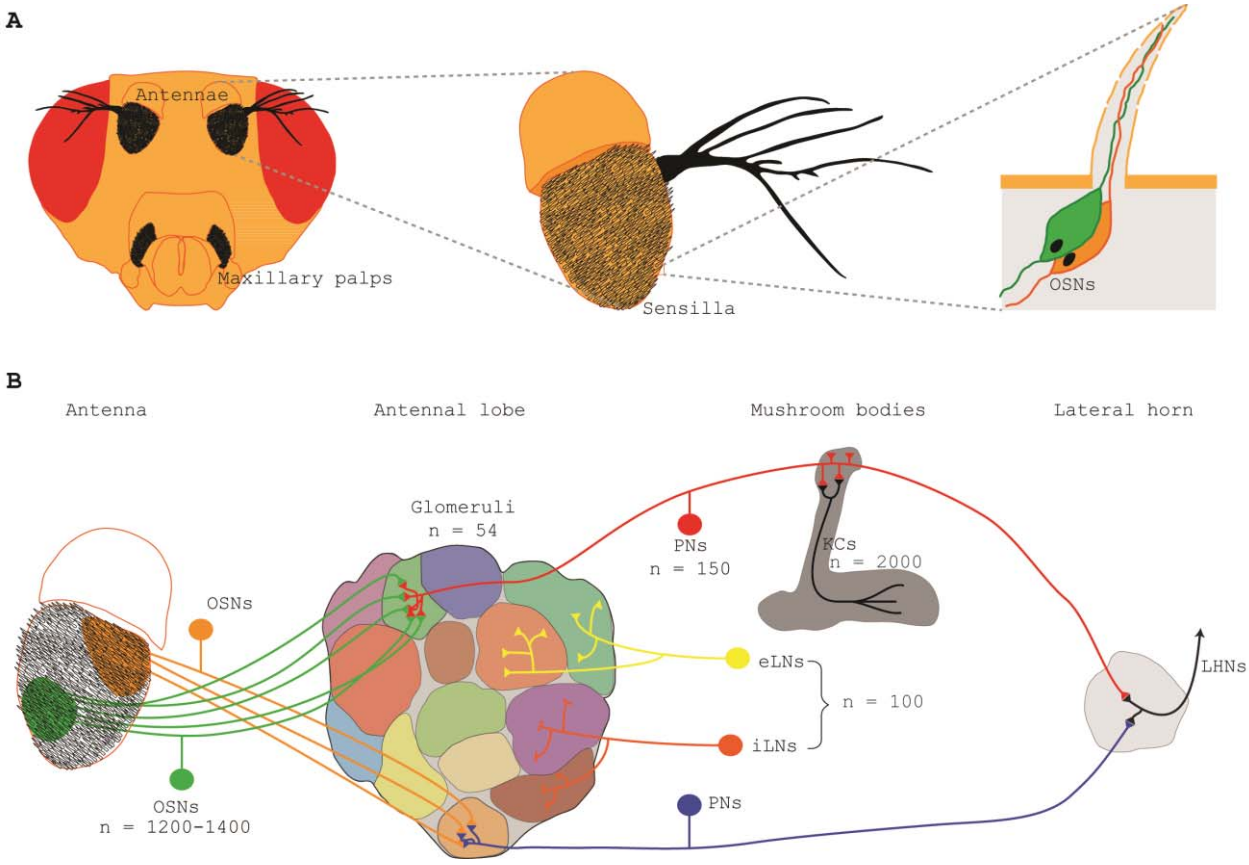

Figure 1|The olfactory system of *Drosophila melanogaster*.

A) Olfactory organs (antennae and maxillary palps) covered with sensory hairs (sensilla), housing dendrites of 1-4 olfactory sensory neurons (OSNs). **B)** Connectome of two OSN types expressing different olfactory receptors, converging into different glomeruli in the antennal lobe, where they interconnect with projection neurons (PNs), transmitting the incoming signal to higher brain centers (mushroom bodies and lateral horn). Signals become further modulated by lateral

interneurons in the antennal lobe (eLNs: excitatory local interneurons, iLNs: inhibitory local interneurons).

The modulated signal is further transmitted *via* PNs to two higher brain centers, the mushroom bodies (MBs) and the lateral horn (LH). Most of the PNs leaving the AL are uniglomerular, which means they are connected to only one specific glomerulus within the AL. These PNs follow two separated tracks. While the lateral antennal lobe tract (lALT) drafts directly to the LH, the medial antennal lobe tract (mALT) branches first in the MBs calyx before it connects to the LH (Stocker, 1994; Vosshall and Stocker, 2007; Ito et al., 2014). A minority of PNs is multiglomerular – transferring the information of more than one glomerulus – and is bunched in the mediolateral antennal lobe tract (mlALT) which terminates only in the LH, bypassing the calyx of the MB (Liang et al., 2013; Parnas et al., 2013; Strutz et al., 2014; Shimizu et al., 2017). The MBs are conventionally described as the center for memory and learning (Davis, 1993; Heisenberg, 2003; Fiala, 2007; Wadell, 2013), but recent investigation revealed another important role of the MBs in controlling the internal state of the fly (Bräcker et al., 2013; Lin et al., 2014). The LH, however, is known as the center of innate behavior in *Drosophila* (Heimbeck et al., 2001). It separates in different clusters coding for innate attraction or innate aversion (Strutz et al., 2014). The publications in this dissertation underline the importance of the LH in fly behavior. Hence, I show how innate behavior of flies can be manipulated using the genetic toolbox of *Drosophila* (Manuscript I). Further we identified innate attractive odors in flies` feces (Manuscript II) and showed that this behavior can also be altered by bacteria (Manuscript III). The searching for food

sources challenges flies in a special way. They have to evaluate odor mixtures of attractive and aversive odors to decide whether it is worth to go for it or not (Manuscript IV). At the end it is astonishing that even attraction towards complex odor blends like fruits can be broke down to innate attraction to single odors within (Manuscript V).

The complexity of the fly´s olfactory system indicates its important role. One part of behavior that is strongly impaired without olfaction is communication between two individuals.

Intraspecific connections: It´s all about communication

First of all I want to define the term intraspecific communication. The syllable "*intra*" means "*within*", so intraspecific communication summarizes all signals (verbal, non-verbal or chemical) that are used by conspecifics to exchange information, e.g. bees waggle dance or the bird song.

One of the best known systems for intraspecific communication is the pheromone system. It is almost omnipresent in the animal kingdom and can be found e.g. in bacteria (Saunders, 1978), insects (Wada-Katsumata et al, 2015), and mammals (Stowers and Kuo, 2015). Especially sex pheromones can become a key factor for reproduction within a species (Butenandt and Hecker, 1961; Hansson et al., 1991; Hildebrand, 1995; Dweck et al., 2015; Hsieh et al., 2017). There are, however, different kinds of pheromones, e.g. bees use alarm pheromones in case of an attack (Nouvian et al., 2016) and ants are known for their usage of trail pheromones (Czaczkes et al., 2015). The most commonly

known group of pheromones is the sex pheromone group. Usually females release pheromones to attract conspecific males over a longer distance (Hildebrand, 1995). Badeke et al. (2016) demonstrated that this signal is strong enough to overcome the surrounding "odor-noise" of the moths' environment. While insects detect both food odors and pheromones with the same olfactory organ (antennae), mammals developed a special organ only dedicated to pheromone detection – the vomeronasal organ (VNO; Stowers and Kuo, 2015). Also humans show an anlage for the VNO, but lose this during the embryonic development (Wackermannova et al., 2016). Furthermore there is evidence that even algae (Hallmann et al., 1998) and fungi (Davey, 1992) use sex pheromones for intraspecific communication. The longest known fly pheromone is cis-vaccenyl acetate (cVA; Bartelt et al., 1985) which is exclusively produced by males and induces a sexual dimorphic response *via* the pheromone receptor OR67d. While females show an enhanced copulation receptivity when the courting male carries a high amount of cVA (Kurtovic et al., 2007), other males show a higher aggression potential in the presence of cVA (Wang and Anderson, 2010). Furthermore males transfer this pheromone during mating on females, which functions as an anti-aphrodisiac (Antony and Jallon et al., 1981). Two years ago more copulation and attraction pheromones in *Drosophila melanogaster* were revealed (Dweck et al., 2015). They could identify three additional compounds, methyl laurate (ML), methyl myristate (MM) and methyl palmitate (MP). While the pheromone receptor OR88a responds to all three identified compounds, OR47b responds only to ML. Furthermore this study revealed the complexity and sensibility of flies` pheromone system, showing that although both olfactory receptors are activated by ML, the behavioral output is totally different.

Obviously Or88a governs aggregation, while OR47b is involved in inducing mating behavior.

The strong predictable response of an animal to its sex pheromone paves the road for misuse by other species, which alter the host system for their own advantage.

Interspecific connections: If the relationship is one-sided

In general there are three different possibilities of interspecific relationships in nature. In symbiosis, both interacting partners get an advantage from this relationship. The most prominent and eponymous (Anton de Bary, 1878) representative for the first form are lichens. While the algae produce carbohydrates through photosynthesis, fungi provide water and mineral nutriment. The second way of building a relationship is called probiosis. This gives an advantage to one of the both participants without benefitting or harming the other. An example is the special relation between big fishes like sharks and *Echeneidae*. Latter adhere to their hosts and use them as a means of transport (Britz and Johnson, 2012).

If one participant benefits from his tie to the other and the other has a disadvantage in this relationship, then one defines it as parasitism. When introducing the term “symbiosis” to science, Anton de Bary also included parasitism in his definition. Today it is considered as a different and, hence, the third possibility of an interspecific relationship. Examples can be found over all taxa. E.g. female mosquitoes suck the blood from vertebrate hosts to use the nutrients for their own egg production (Lyimo et al., 2017). Furthermore these insects

are often vectors for severe diseases like malaria or dengue (de Boer et al., 2017; Trewin et al., 2017). However, parasites are not only found in the animal or plant kingdom. Also bacteria can parasite a host and alter its metabolism or behavior to their own advantage (Spagnoli et al., 2016; Sanchez et al., 2008; Mann et al., 2012). It also occurs that pathogens infect and manipulate the physiology and ultimately the behavior of alternate hosts to get a better chance infecting their real host.

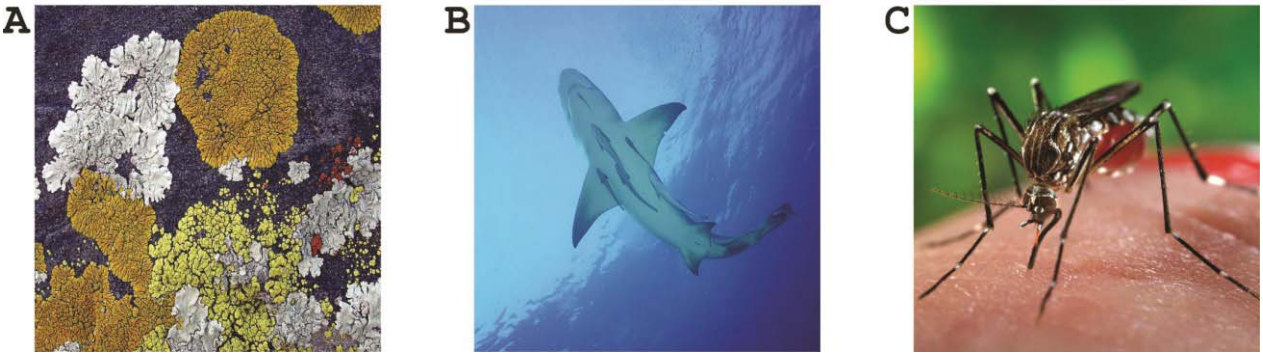

Figure 2|Interspecific relationships

Examples for **A)** symbiosis: Lichens consisting of algae and fungi, with algae producing carbohydrates through photosynthesis and fungi providing water and nutrients. (image source: https://ferrebeekeeper.files.wordpress.com/2011/03/lichen27.jpg) **B)** Probiosis: *Echeneidae* adhered to a shark and benefiting from faster movement without affecting their host. (image source: http://www.tierchenwelt.de/images/stories/fotos/fische/barschartige/schiffshalter/schiffshalter_hai.jpg) **C)** Parasitism: Female mosquito leeching the blood of its host to use the nutrients for egg production. (image source: http://www.the-scientist.com/images/Nutshell/December2016/16740-close-up-of-a-mosquito-feeding-on-blood-pv.jpg)

Good examples are the tactics of *Toxoplasma gondii* whose ultimate hosts are cats. The olfactory receptors of an infected mouse responsible for the detection of compounds of cat urine are affected due to infection with *Toxoplasma* (Ingram et al., 2013). This loss of function reduces fear and flight behavior of infected mice which leads to a higher probability for these animals to get caught and eaten by a cat.

However, in Manuscript III we show evidence for another example of host-alternating parasitism. We infected *Drosophila melanogaster* with different naturally occurring pathogens and observed how the body odors of these flies change the behavior of their not infected conspecifics. Healthy flies were strongly attracted to infected flies and their feces. This attraction lead to close contact resulting in the transfer of pathogens to formerly healthy flies. We found that the pathogen induced this increased attraction by increasing the pheromone emission rate of sick flies.

Aims of this thesis

With my thesis I am able to highlight the importance of appropriate controls when using the genetic toolbox in *Drosophila melanogaster*. Furthermore I disclose new insights in intraspecific and interspecific communication in *Drosophila melanogaster*.

Manuscript I deals with the use of the genetic toolbox of *Drosophila melanogaster* in different behavior paradigms and its limits. We could show that the efficiency of single effectors that should shut down parts of the flies' olfactory system, depends strongly on the expression level of these genes and the behavior setup that is used to test the flies' behavior.

In Manuscript II we show that pheromones that recently had been identified in *D. melanogaster* (Dweck et al., 2015) appear in high amounts in the frass of the flies. Frass, therefore provokes aggregation of flies. This aggregation obviously is species specific, as GC-MS analysis revealed that the closer fly species are related the more alike is the composition of their frass.

Furthermore, in Manuscript III we could show that pathogens increase the pheromone production of *Drosophila melanogaster*. This enhancement leads to a higher attraction and aggregation of conspecifics.

Moreover, with Manuscript IV I took part in a study revealing that avoidance of odorant mixtures of attractive and aversive odors is ratio dependent and relies also on the inner status of the fly.

Finally I contributed in Manuscript V in a study investigating differences in fruit preferences between larvae and adult flies of *Drosophila melanogaster*.

Manuscript Overview

Manuscript I

Potencies of effector genes in silencing odor-guided behavior in *Drosophila melanogaster*

Tom Retzke, Michael Thoma, Bill S. Hansson, Markus Knaden

Journal of Experimental Biology, 2017, 220:1812-1819

In this study, we investigated the potencies of several effector genes – when expressed in olfactory sensory neurons – to abolish odor-guided behavior in three different bioassays. We found that two of the tested effectors are capable of mimicking the Orco mutant phenotype in all of our behavioral paradigms. In both cases, the effectiveness depended on effector expression levels, as full suppression of odor-guided behavior was observed only in flies homozygous for both Gal4-driver and UAS-effector constructs. Interestingly, the impact of the effector genes differed between chemotactic assays and anemotactic assays.

Author contributions

Designed experiments: TR (50%), MT, BSH, MK

Performed Experiments: TR (100%)

Analyzed experiments: TR (95%), MT

Wrote the manuscript: TR (50%), MT, MK, BSH

Manuscript II

Adult Frass Provides a Pheromone Signature for *Drosophila* Feeding and Aggregation

Ian W. Keesey, Sarah Koerte, Tom Retzke, Alexander Haverkamp, Bill S. Hansson, Markus Knaden

Journal of Chemical Ecology, 2016, 42:739-747

In this study we show that *Drosophila* adults are able to recruit conspecifics to a food source by covering it with fecal spots, and that this behavior is mediated *via* olfactory receptors. In addition we demonstrate that also adult feeding is increased in the presence of frass.

Author contributions

Designed experiments: IWK, BSH, MK

Performed Experiments: IWK, SK, TR (15%)

Analyzed experiments: IWK, SK, TR (15%), AH

Wrote the manuscript: IWK, BSH, MK

Manuscript III

Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

Ian W. Keesey, Sarah Koerte, Mohammed A. Khallaf, Tom Retzke, Aurelien Guillou, Ewald Grosse-Wilde, Nicolas Buchon, Markus Knaden, Bill S. Hansson

Nature Communications, 2017, 8:265

This study provides evidence that infection with pathogenix bacteria alters the social communication system of *Drosophila melanogaster*. Infected flies showed an increased production of aggregation pheromones and for this reason were more attractive for conspecifics. We show that this alteration can be beneficial for the pathogenic bacteria.

Author contributions

Designed experiments: IWK, BSH, NB, MK

Performed Experiments: IWK, SK, TR (15%)

Analyzed experiments: IWK, SK, TR (15%), MAK

Wrote the manuscript: IWK, BSH, NB, MK

Manuscript IV

Odor mixtures of opposing valence unveil interglomerular crosstalk in *Drosophila* antennal lobe

Ahmed A. M. Mohamed, Tom Retzke, Sudeshna Das, Bill S. Hansson, Markus Knaden and Silke Sachse

In preparation as a research article in Neuron

In this study we show that crosstalk between glomeruli coding for opposing valence in the antennal lobe of *Drosophila melanogaster*. We observed ratio-dependent attraction of flies towards binary mixtures of an attractant and a repellent. This behavior was mirrored by activation patterns found in the antennal lobe.

Author contributions

Designed experiments: AAMM, TR, MK, SS

Performed Experiments: AAMM, TR (10%)

Analyzed experiments: AAMM, TR (10%)

Wrote the manuscript: AAMM, MK, SS, TR(5%)

Manuscript V

Logic Behind Differences in Food Preferences Between Larval and Adult *Drosophila*

Hany K. M. Dweck, Shimaa A. M. Ebrahim, Tom Retzke, Veit Grabe, Jerrit Weißflog, Markus Knaden, Bill S Hansson

Manuscript submitted to Neuron

In this study we show behaviors generated by larval and adult flies to 34 fruit-headspace extracts, and find that larvae and adults show different preferences when exposed to these fruit extracts. Furthermore, we provide a functional analysis of the full repertoire of the olfactory system in larval and adult flies. We find that 90 and 53 percent of the olfactory system in larval and adult flies, respectively, are involved in evaluating these food resources. Finally, we find that the number of receptor/neuronal responses correlates strongly positively with behavioral output in larvae, and correlates weakly negatively in adult flies.

Author contributions

Designed experiments: HKMD, MK, BSH

Performed Experiments: HKMD, SAME, TR (30%), JW

Analyzed experiments: TR (30%), HKMD, SAME, VG, JW

Wrote the manuscript: HKMD, MK, BSH

Manuscript I

Potencies of effector genes in silencing odor-guided behavior in *Drosophila melanogaster*

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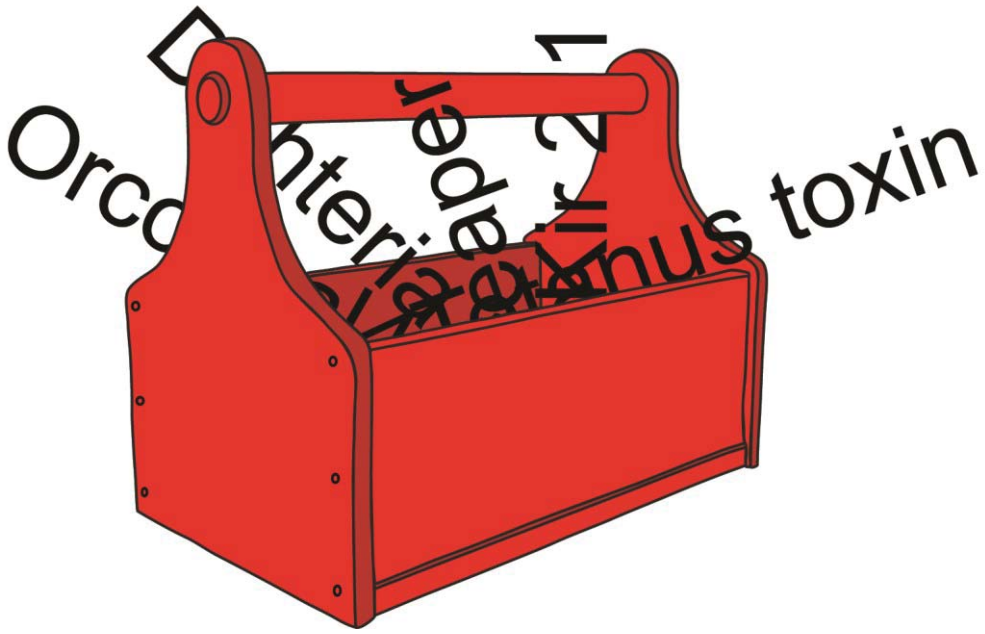
Journal of Experimental Biology, 2017, 220:1812-1819



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RESEARCH ARTICLE

Potencies of effector genes in silencing odor-guided behavior in *Drosophila melanogaster*

Tom Retzke‡, Michael Thoma*,‡, Bill S. Hansson§ and Markus Knaden§,¶

ABSTRACT

The genetic toolbox in *Drosophila melanogaster* offers a multitude of different effector constructs to silence neurons and neuron populations. In this study, we investigated the potencies of several effector genes – when expressed in olfactory sensory neurons (OSNs) – to abolish odor-guided behavior in three different bioassays. We found that two of the tested effectors (*tetanus toxin* and *Kir2.1*) are capable of mimicking the *Orco* mutant phenotype in all of our behavioral paradigms. In both cases, the effectiveness depended on effector expression levels, as full suppression of odor-guided behavior was observed only in flies homozygous for both Gal4-driver and UAS-effector constructs. Interestingly, the impact of the effector genes differed between chemotactic assays (i.e. the fly has to follow an odor gradient to localize the odor source) and anemotactic assays (i.e. the fly has to walk upwind after detecting an attractive odorant). In conclusion, our results underline the importance of performing appropriate control experiments when exploiting the *D. melanogaster* genetic toolbox, and demonstrate that some odor-guided behaviors are more resistant to genetic perturbations than others.

KEY WORDS: *Drosophila*, Behavior, Effector genes, Olfaction

INTRODUCTION

Much of the success of *Drosophila melanogaster* as a model organism in neuroscience is attributable to its genetic tractability. Binary expression systems such as the Gal4/UAS system can be used to drive expression of specific effector genes to genetically defined target neuron populations, allowing visualization of morphology and activity, and artificial activation and/or silencing (Venken et al., 2011). This way, the contribution of genetically identifiable neuronal subpopulations of sensory systems to the overall perception and evaluation of a given sensory stimulus can be studied in detail.

Drosophila melanogaster detects odors using an array of olfactory sensory neurons (OSNs) located in sensory hairs termed olfactory sensilla. Olfactory sensilla are located on two types of head appendages, the antennae and the maxillary palps (Stocker, 1994; Vosshall and Stocker, 2007). Most OSNs are activated by more than one odorant and most monomolecular odorants, and, more importantly, natural 'odors' consisting of several monomolecular odorants typically activate multiple OSN classes (de Bruyne et al., 1999, 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Pelz et al., 2006; Silbering et al., 2011; Dweck et al., 2016). Therefore, the identity of most odors is encoded in the combinatorial activity of the OSN population as a whole (Malnic et al., 1999). Nevertheless, recent evidence suggests that the innate hedonic valence of odors can already be predicted on the basis of the identity of OSNs the odors activate (Ai et al., 2010; Dweck et al., 2013, 2015a,b; Grosjean et al., 2011; Knaden et al., 2012; Kurtovic et al., 2007; Min et al., 2013; Ronderos et al., 2014; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004; Thoma et al., 2014). It is therefore tempting to remove specific OSN populations from the activity pattern and in this way to investigate their contribution to the overall valence of a given odor.

As a rule with few exceptions, each OSN expresses one type of ligand-binding chemoreceptor, which defines its ligand specificity (Couto et al., 2005; Fishilevich and Vosshall, 2005). With one exception, i.e. the CO_2 detection system comprising two gustatory receptors (GRs) (Jones et al., 2007; Kwon et al., 2007), all antennally expressed olfactory chemoreceptors in *D. melanogaster* belong to one of two gene families, the evolutionarily ancient ionotropic receptors (IRs) detecting mainly – but not exclusively – hydrophilic chemicals (Abuin et al., 2011; Ai et al., 2010; Benton et al., 2009; Grosjean et al., 2011; Min et al., 2013; Silbering et al., 2011) and the insect-specific odorant receptors (ORs) (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). All *D. melanogaster* ORs rely on the ubiquitously expressed co-receptor *Orco* for intracellular trafficking (Larsson et al., 2004) and signal transduction (Sato et al., 2008; Wicher et al., 2008), and OR-expressing OSNs lacking the *Orco* protein are generally unresponsive to odors. This genetic make-up of the OR-based olfactory system of *D. melanogaster* with variable ligand-binding ORs and a common co-receptor is ideally suited to investigate the effect of the removal of an OSN population expressing a particular OR on odor evaluation. Even in the absence of an observable effect of silencing a specific OSN population under control of the promotor of the odor-binding OR, efficiency of silencing can be controlled by targeting the silencing effector gene to the whole OR-expressing OSN population under control of the *Orco* promotor.

There are several ways to genetically silence neurons in *D. melanogaster*. Neurons can be ablated by expressing bacterial toxins or pro-apoptotic genes, synaptically silenced using *tetanus toxin* (*TeTx*) or a dominant negative form of dynamin (*shibire^{ts}*), or electrically silenced by ectopic expression or RNAi-induced downregulation of ion channels (Venken et al., 2011). Here, by targeting all OR-expressing OSNs, we test the efficiency of the expression of *diphtheria toxin* (*DTA*; Han et al., 2000), the pro-apoptotic gene *reaper* (*rpr*; Zhou et al., 1997), *TeTx* (Sweeney et al., 1995) and the potassium channel *Kir2.1* (Baines et al., 2001; Paradis et al., 2001) in OSNs using the Gal4/UAS system

Max Planck Institute for Chemical Ecology, Department of Evolutionary Neuroethology, Hans-Knoell-Straße 8, Jena 07745, Germany.
*Present address: The New Zealand Institute for Plant & Food Research Limited, Private Bag 92169, Auckland 1142, New Zealand.
§These authors share senior authorship
‡These authors contributed equally to this work

¶Author for correspondence (mknaden@ice.mpg.de)

M.K., 0000-0002-6710-1071

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(Brand and Perrimon, 1993) in suppressing odor-guided behavior in three different behavioral bioassays, a two-choice trap assay, the Flywalk (Steck et al., 2012) and an open-field arena. Similar to the observation by others who investigated the efficiency in the motor system and the mushroom body (MB) (Thum et al., 2006), we found that the effector genes differed in their potencies of abolishing odor-guided behavior. *DTA* and *rpr* did not abolish odor-guided behavior in any of the bioassays. *Kir2.1* and *TeTx* were partially effective, and their potency depended on the type of bioassay and expression level. Importantly, our results show that it is absolutely crucial to perform appropriate control experiments when using the *D. melanogaster* genetic toolbox to dissect the contribution of individual neuron populations to behavior.

MATERIALS AND METHODS

Flies

Flies were reared on standard cornmeal medium at 23°C, 70% relative humidity under a 12 h:12 h light:dark regime. All experimental flies were 6–8 days old and were starved, but not water-deprived, for 24 h before the experiments.

We used Orco-Gal4 to drive expression of the effector genes *rpr*, *DTA*, *TeTx* and *Kir2.1* specifically in Or-expressing OSNs (for details on original genotypes and sources, see Table 1). In addition, we performed experiments in Canton S wild-type and *Orco[2]* mutant flies. All Gal4 and UAS lines were backcrossed to w^{1118} flies to reduce variability conferred by the genetic background.

Chemicals

All monomolecular odorants were purchased from Sigma-Aldrich or FLUKA at the highest purity commercially available and diluted in mineral oil (also Sigma-Aldrich). In addition, we used commercially available balsamic vinegar in Flywalk and open-field arena experiments.

Trap assay

Trap assays were performed as previously described (Knaden et al., 2012) (Fig. 1A). The testing chamber consisted of a plastic box (length 10.5 cm, width 7.5 cm, height 9.5 cm) containing two traps constructed from smaller plastic vials (diameter 3.1 cm, height 4.3 cm). Flies could enter the traps through the cut end of a pipette tip, but once inside could not leave the traps. One of the cups contained a 0.2 ml PCR reaction tube containing a round piece of filter paper (diameter 1 cm) loaded with 100 µl of a 10^{-3} dilution of ethyl acetate (ETA) in mineral oil as an attractive odor source. The other trap served as a control, in which the filter paper was loaded with mineral oil only. Directly after preparation of the traps, cohorts of 40–80 flies of mixed sex were introduced into the testing box and allowed to choose between the traps for 24 h at 23°C and 70% relative humidity in complete darkness. Attraction was scored by calculating an attraction index (AI) as:

$$\mathrm{AI} = (n_{\mathrm{odor}} - n_{\mathrm{control}})/n_{\mathrm{total}}, \quad (1)$$

where n_{odor} is the number of flies in the odor trap, n_{control} is the number of flies in the control trap and n_{total} is the total number of flies tested in the experiment. Positive AI values indicate attraction, negative values indicate repulsion.

Table 1. List of the used transgenic fly lines

Fly line	BL no.	Genotype	Source
Orco$^{-/-}$	23130	yw; +; *Orco*^{2}	Bloomington *Drosophila* Stock Center
UAS-*Kir2.1*	6596	w; P{w[+mC]=UAS-Hsap\KCNJ2.EGFP}1/(CyO); +	Bloomington *Drosophila* Stock Center
UAS-*Diphtheria toxin*	25039	w; P{w[+mC]=UAS-Cbbeta\DT-A.I}18/CyO; +	Bloomington *Drosophila* Stock Center
UAS-*Tetanus toxin*	28837	w; P{w[+mC]=UAS-TeTxLC.tnt}E2; +	Bloomington *Drosophila* Stock Center
UAS-*reaper*	5824	w; P{w[+mC]=UAS-rpr.C}14; +	Bloomington *Drosophila* Stock Center
Orco-Gal4 driver line		yw; +; *Orco*->Gal4	Andre Fiala

Flywalk

Flywalk experiments were performed as previously described (Steck et al., 2012; Thoma et al., 2014). Briefly, 15 individual flies, starved for 24 h, were placed in parallel aligned glass tubes and their positions recorded under red-light conditions (λ=630 nm) over a period of ∼8 h. Flies were continuously exposed to a humidified airflow (∼20°C, ∼75% relative humidity) of 0.3 liters min^{-1} (20 cm s^{-1} in the glass tubes). Repeated odor pulses (inter-stimulus interval 90 s) were released from a multicomponent stimulus device (Olsson et al., 2011) loaded with 100 µl of odor dilutions in mineral oil. Responses were calculated as the mean distance flies covered within 4 s of encounter with the odor pulse.

Open-field arena

The open-field arenas consisted of rectangular polystyrene Petri dishes (125 mm to each side and 16 mm high) with a central hole (diameter 7 mm) in the lid. The hole was occluded with gauze from the inside and a round piece of filter paper (diameter 10 mm) from the outside. This way, flies could not physically contact the odor that was pipetted on the filter paper. The arena was illuminated by red LEDs (λ=630 nm) from above and monitored using a webcam (HD Pro Webcam C920, Logitech, Lausanne, Switzerland) from below.

At the beginning of an experimental session, a single female fly was introduced into the arena and allowed to habituate to the new environment for 5 min. Afterwards, 10 µl of distilled water was carefully added to the filter paper under red-light conditions and without mechanical disturbances, and the fly was recorded at 30 frames s^{-1} for 10 min using Media Recorder 2 software (Noldus Information Technology, Wageningen, The Netherlands). Finally, 10 µl of balsamic vinegar was added to the filter paper and the fly was again recorded for another 10 min. Flies were then tracked offline by dynamic background subtraction using EthoVision XT software (Noldus Information Technology). Further analysis was performed using R (www.r-project.org).

For all experiments and corresponding sample sizes, the sample size numbers used in this study (Flywalk, N=15 flies; trap assay, N=8–15 replicates with each 40–80 flies; arena assay, N=20 flies) have been proven to yield significant results.

RESULTS

To investigate the potencies of different effector genes in silencing odor-guided behavior, we expressed the silencers *rpr*, *DTA*, *Kir2.1* and *TeTx* in OSNs under Orco-Gal4 control and examined odor-guided behavior in three different behavioral bioassays. Flies were heterozygous for both Orco-Gal4 and UAS-effector unless mentioned otherwise.

We first examined odor-guided behavior in a simple two-choice trap assay (Fig. 1A). Canton S wild-type flies as well as the parental

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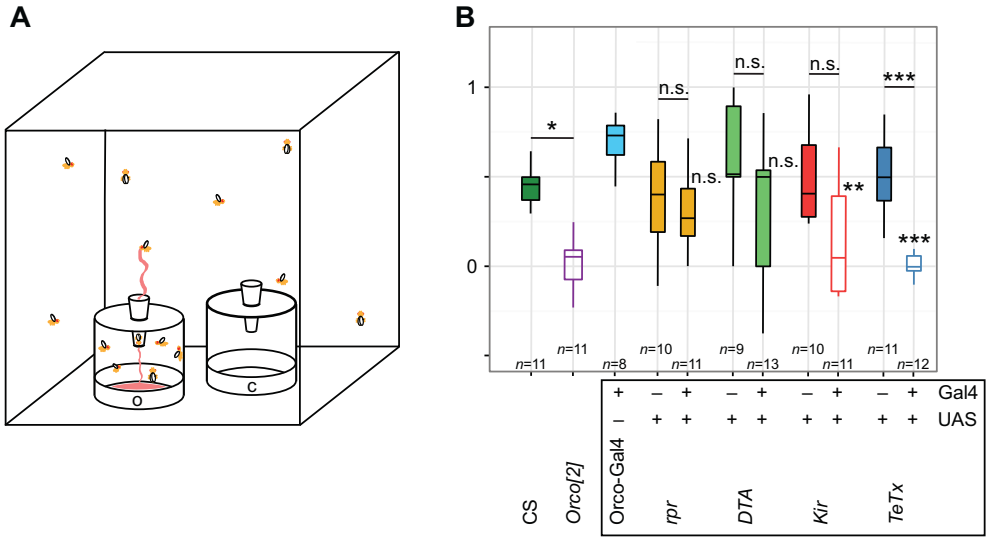

Fig. 1. Efficiency of effector genes in a two-choice trap assay. (A) Schematic representation of the trap assay. Forty to 80 flies are released in a plastic box containing two traps. One of the traps is loaded with odor in mineral oil, the other with the solvent mineral oil. Flies are allowed to choose between traps for 24 h and an attraction index (AI) is calculated. (B) Responses of control lines and flies expressing the effectors under Orco-Gal4 control to a 10^{-3} dilution of ethyl acetate (ETA). Filled boxes indicate statistically significant attraction (P<0.05, one-sample Wilcoxon rank sum test). Asterisks above boxplot show statistical significance between experimental flies and UAS controls, asterisks to the right of the boxes indicate statistical significance between experimental flies and Orco-Gal4 control (Kruskal–Wallis test with Dunn's *post hoc* test for multiple comparisons, P<0.05). CS, Canton S; *rpr*, *reaper*; *DTA*, *diphtheria toxin*; *TeTx*, *tetanus toxin*.

Orco-Gal4 flies were significantly attracted to ETA (Fig. 1B). Importantly, attraction was abolished in *Orco* mutant flies, suggesting that input from *Orco*-expressing OSNs is necessary to induce ETA attraction. Because we expressed the effector genes under Orco-Gal4 control and therefore in the expression pattern of *Orco*, effective silencing by the effector genes should recapitulate this loss of attraction. However, neither *rpr* nor *DTA* abolished attraction when expressed in OSNs (Fig. 1B). In both cases, the AI did not differ between experimental flies and the parental UAS controls. In contrast, attraction was abolished in flies expressing *Kir2.1* and *TeTx* in OSNs (Fig. 1B). However, we did not observe a significant difference between the AIs in *Kir2.1*-expressing flies and the corresponding parental UAS-control flies. This might be due to the low sample size and the generally higher behavioral variability of flies carrying effector constructs. From these experiments, we conclude that different effector genes are differentially effective in silencing *D. melanogaster* OSNs with the most effective one being *TeTx*.

Next, we investigated the effectors' potencies in suppressing odor-guided behavior in the Flywalk assay (Steck et al., 2012) (Fig. 2A). Importantly, in this bioassay, the localization of the odor source does not depend on chemotaxis along a chemical gradient, but, rather, on odor evaluation by the olfactory system and on wind direction as a directional cue for the localization of the odor source (anemotaxis). When presented with a 1-s pulse of the saturated headspace of an attractive 10^{-3} dilution of ethyl acetate in mineral oil, flies responded with instantaneous upwind trajectories, which were absent or only weak when flies were presented with the solvent mineral oil (MOL; Fig. 2B). Similar to the observation in the trap assay experiments, responses to ETA were abolished in *Orco* mutants (Fig. 2C). In addition to ETA, we examined fly behavior towards balsamic vinegar (BVI), methyl acetate (META; 10^{-3} dilution), 2,3-butanedione (BDN; 10^{-3} dilution), trans-2-hexenol (t2H; 10^{-1} dilution) and benzaldehyde (BEA; 10^{-1} dilution). Wild-type flies were significantly attracted by ETA, BVI, META and BDN, whereas t2H was behaviorally neutral and BEA responses were significantly lower than responses towards MOL. In contrast, the attraction induced by ETA, META and BDN as well as the repulsion induced by BEA were abolished in *Orco* mutant flies (Fig. 2D). *Orco* mutant flies retained a residual attraction towards BVI, which is probably conferred by the detection of acetic acid via IRs. In addition, the *Orco* mutant flies acquired attraction towards t2H, which, importantly, is not a false positive in this dataset, but highly reproducible in other datasets (data not shown). We included this odor, because we reasoned that efficient silencing of *Orco*-expressing OSNs should also recapitulate this gain of attraction toward t2H.

As already observed in trap assays, *DTA* and *rpr* also failed to abolish odor-guided behavior in most cases in the Flywalk paradigm (Fig. 2E,F). With the exception of the responses towards ETA, which were abolished in *Orco*-Gal4/UAS-*rpr* flies, all attraction responses were retained in flies expressing *DTA* and *rpr*. Also, in most cases in which flies expressing the two effectors differed in their responses from one of their parental control flies, responses were statistically indistinguishable from the other parental line (Fig. S1A,B). Similar to *DTA* and *rpr*, expressing *Kir2.1* in *Orco*-expressing OSNs failed to abolish any of the attractive responses and the aversion of BEA, whereas the gain of attractiveness of t2H observed in *Orco* mutant flies could be recapitulated by this manipulation, but not in parental controls (Fig. 2G, Fig. S1C). Importantly, *Kir2.1* was partly efficient in modifying attractant responses, as it significantly decreased responses compared with both parental control strains for BVI and BDN. Expressing *TeTx*, in contrast, abolished attraction towards META and reduced, but not abolished, responses towards BVI and BDN compared with parental controls, and failed to recapitulate the gain of attraction towards t2H (Fig. 2H, Fig. S1D). Also, the aversion induced by BEA was suppressed, which is

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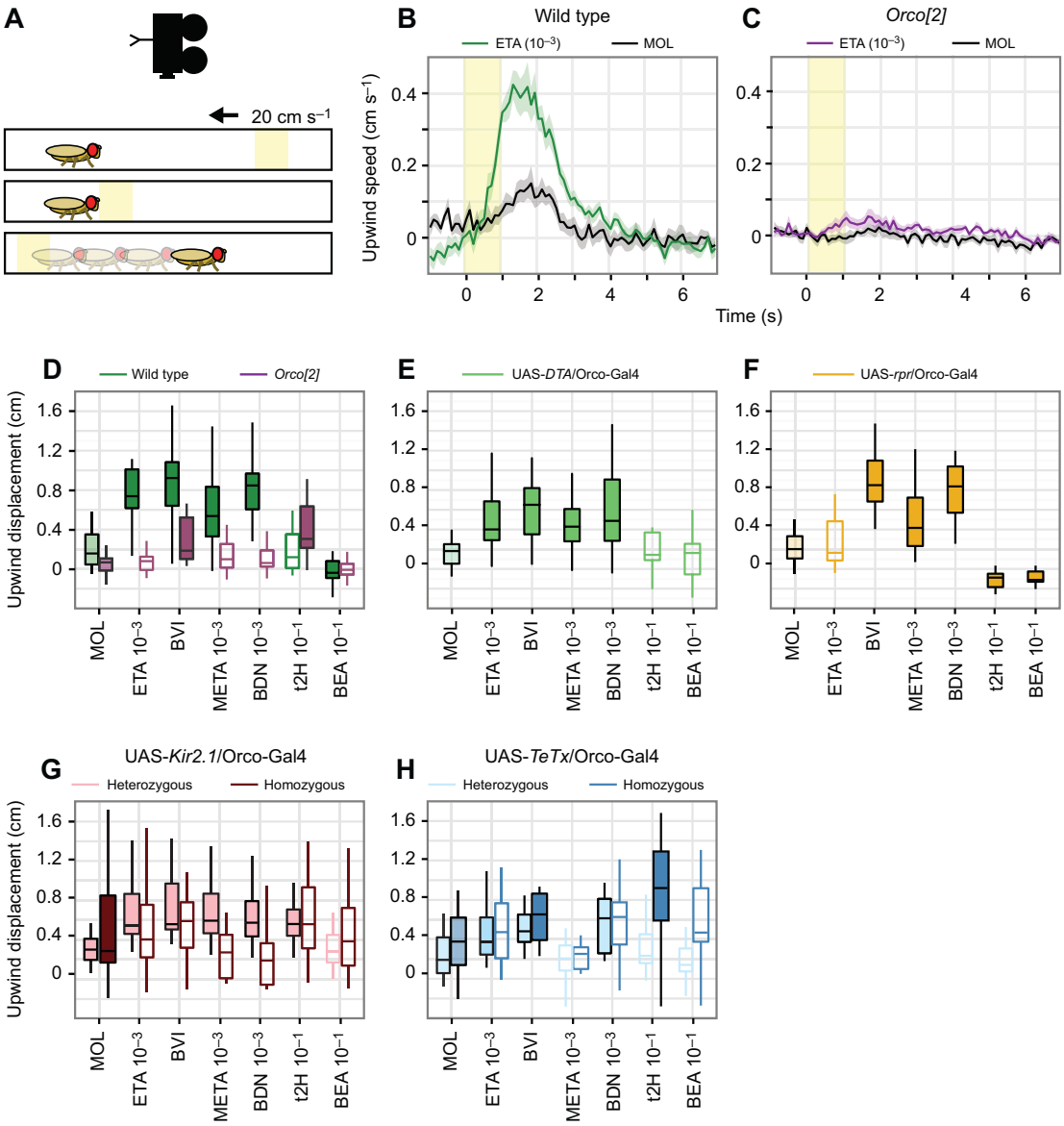

Fig. 2. Efficiency of effector genes in the Flywalk paradigm. (A) Schematic representation of the Flywalk paradigm. Individual flies are situated in small glass tubes and continuously monitored by an overhead camera. Odors are added to a constant airflow and fly movement after odor encounter is analyzed. (B) Speed trajectories after encounter with a 1 s pulse of a 10^{-3} dilution of ethyl acetate (ETA, green) and the negative control mineral oil (MOL; black; mean± s.e.m.; *n*=15 flies) in wild-type flies. (C) Speed trajectories after encounter with a 1 s pulse of a 10^{-3} dilution of ETA (violet) and the negative control MOL (black) in *Orco* mutant flies (mean±s.e.m.; *n*=15 flies). (D) Odor-induced upwind displacement in CS wild-type and *Orco* mutant flies to a set of odors. In D–H, filled boxes indicate statistically significant differences from the negative control MOL (transparent colors; Wilcoxon signed rank test, $P<0.05$, *n*=15 flies). (E,F) Odor-induced upwind displacement in flies heterozygously expressing *DTA* (E) and *rpr* (F) in the expression pattern of *Orco*. Note residual responses compared with *Orco* mutant flies shown in D. (G,H) Odor-induced upwind displacement in flies expressing *Kir2.1* (G) and *TeTx* (H) in the expression pattern of *Orco*. Lighter boxes show responses of flies expressing *Kir2.1* (G) or *TeTx* (H) heterozygously, darker boxes show responses of flies expressing *Kir2.1* (G) or *TeTx* (H) homozygously.

probably attributable to decreased responses towards the negative control compared with control genotypes, which can also be observed in *Kir2.1*-expressing flies (Fig. 2GH, Fig. S1CD). In summary, although *Kir2.1* and *TeTx* (and for one odor also *rpr*) reduced some of the odor-induced responses in the Flywalk paradigm, the neuronal silencing induced by the tested effectors was not absolute. None of the effectors succeeded in fully recapitulating the Orco mutant phenotype, at least not when heterozygously expressed. Because *TeTx* and potentially *Kir2.1* were effective in the trap assay and also displayed some effect in initial Flywalk experiments, we proceeded to investigate whether an increase in transgene expression levels by double-homozygous expression would increase the efficiency of these two effectors.

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Homozygously expressed *Kir2.1* was very efficient in abolishing odor-guided behavior in the Flywalk. *Kir2.1*-expressing flies did not show any statistically significant response to the presented odorants in the Flywalk paradigm (Fig. 2G). However, homozygous expression of *Kir2.1* could not fully recapitulate the *Orco* mutant phenotype, because responses towards BVI and t2H were also abolished. In contrast, homozygous expression of *TeTx* in *Orco*-expressing OSNs could fully recapitulate the *Orco* mutant phenotype (including residual responses to BVI and novel response to t2H; Fig. 2H).

So far, we showed that even effectors that successfully abolish odor-guided attraction in the trap assay may fail to do so in the Flywalk paradigm. What could be the reason for the differences in potencies observed in the different bioassays? Essentially, the two bioassays differ in two aspects: (1) in the trap assay, we tested cohorts of flies in contrast to individual flies in Flywalk, and (2) in the trap assay flies rely on chemotaxis along an odor gradient in contrast to the anemotactic odor source localization in Flywalk. To identify the reason for the conflicting results obtained so far, we next examined odor-guided behavior in a single-fly chemotactic assay similar to that described by others (Zaninovich et al., 2013). In this paradigm, we released individual flies in a square arena with a central odor source, recorded their positions and analyzed their distance from the central odor source (Fig. 3A). Because responses to single odorants are not very strong in this assay, we used balsamic vinegar as an attractant and distilled water as a negative control in these experiments.

When presented with water as a central odor source, CS flies typically spend most of the time at the edges of the arena and otherwise explore the whole arena without displaying spatial preferences (Fig. 3B). When presented with balsamic vinegar, in contrast, CS flies still spend a significant amount of time at the arena edges, but otherwise display intensive search behavior in the arena center (Fig. 3C). To analyze this observation quantitatively, we calculated the flies' mean distance from the arena center for both water and vinegar. Thus we observed that wild-type flies are attracted to the balsamic vinegar. Similar to wild-type flies, *Orco* mutant flies were also attracted to balsamic vinegar in this assay (Fig. 3D). In a more detailed analysis, we found that *Orco* mutants, compared with CS, spent significantly less time within 2 cm around the odor source when presented with vinegar (P=0.006, Wilcoxon rank sum test, n=20; Fig. S2B,C), suggesting that they are indeed impaired in their fine-scale search behavior, although they are still able to detect the odor source, probably detecting acetic acid using the IR-dependent olfactory subsystem.

We next tested flies heterologously expressing the different effector genes in this assay. According to the results obtained in the trap assay and Flywalk experiments, we did not observe any difference between *DTA*-expressing flies and their corresponding parental controls. All tested animals spent an equal amount of time within 2 cm of the odor source (Fig. 3E). The same was found for flies expressing *rpr* under *Orco*-Gal4 control. Although *Kir2.1* by trend abolished attraction in the trap assay experiment

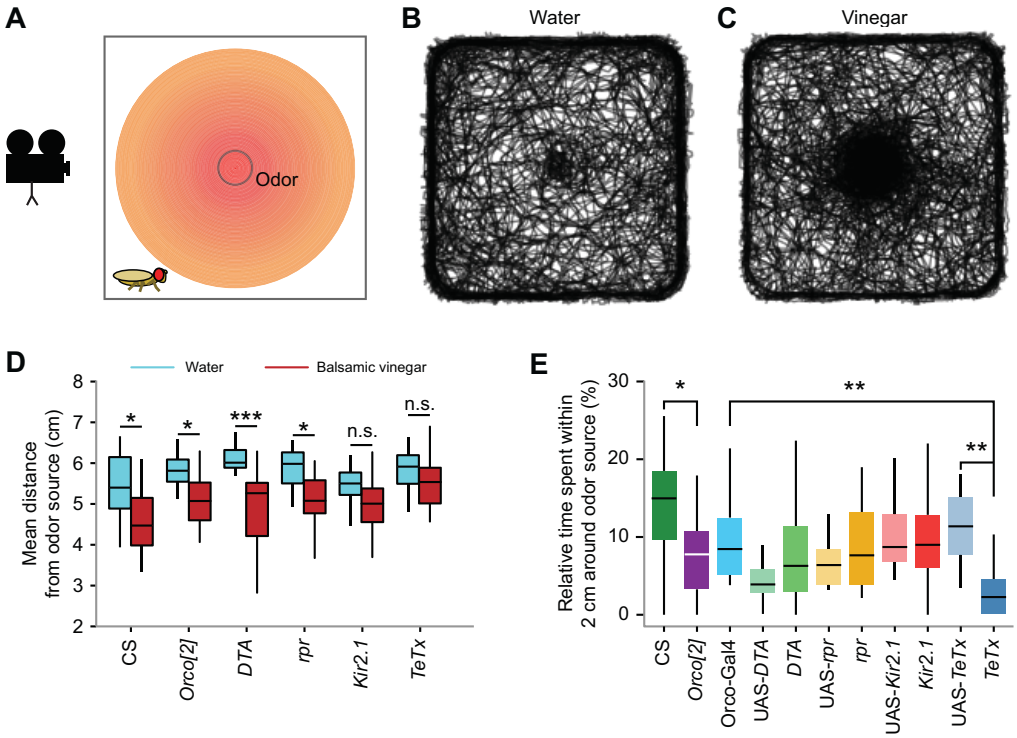

Fig. 3. Efficiency of effector genes in an open-field arena. (A) Schematic representation of the open-field arena. Individual flies are released in a square arena with a central odor source and their positions are recorded for 10 min. The odor source cannot be contacted physically by the fly. (B,C) Overlay of 20 fly trajectories when presented with water (B) and balsamic vinegar (C). Note increased search behavior in the central zone in C. (D) Mean distance from odor source of CS, *Orco[2]*, *DTA*-expressing, *rpr*-expressing, *Kir2.1*-expressing and *TeTx*-expressing flies. Water control is indicated by blue boxes, balsamic vinegar indicated by red boxes. Asterisks indicate significant differences (*P<0.05; **P<0.01; ***P<0.001; Friedman test; n=20 flies per genotype). (E) Relative time the tested flies spent within 2 cm around odor source (balsamic vinegar). Asterisks indicate significant differences (*P<0.05; **P<0.01; Wilcoxon rank sum test, Bonferroni-corrected; n=20 flies per genotype).

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and – if homozygously expressed – did so significantly in Flywalk experiments, we could not find any difference between experimental flies and parental controls in the open-field arena (Fig. 3E). This leads to the conclusion that *Kir2.1* expression is not able to reproduce the *Orco* mutant phenotype and therefore fails to completely abolish odor-guided behavior in this single-fly chemotactic bioassay. In contrast, *TeTx*-expressing flies showed a significantly lower attraction towards balsamic vinegar compared with parental controls (Fig. 3E). Experimental flies were not attracted to the water control or balsamic vinegar. Although they explored the arena and occasionally also passed the central odor source, they did not show any search behavior that is comparable to the behavior observed in the other genotypes (not shown).

We conclude that only two of the constructs we tested fully recapitulated the *Orco* mutant phenotype in all bioassays. However, when expressed heterozygously, both *rpr* and *DTA* failed to induce any expression-specific effect in any of the paradigms, while *Kir2.1* slightly reduced attraction in trap assays, but not in Flywalk or the open-field arena. *TeTx* appears to be the most efficient genetic tool for silencing OSNs, although it also failed to completely abolish odor-guided behavior in the *Flywalk* paradigm in the heterozygous expression regime. Only when expressed homozygously were both *TeTx* and *Kir2.1* able to abolish odor-guided behavior in Flywalk experiments, where *TeTx* fully recapitulated the *Orco* mutant phenotype. In addition, our results in combination suggest that the different potencies we observed in the three bioassays may at least be partially explained by the different demands on olfactory processing between chemotaxis and anemotaxis.

DISCUSSION

Our main objective in this study was to identify a genetic tool that is capable of reliably silencing single OSN populations in a large-scale approach to investigate the contribution of individual processing channels of the fly olfactory system to odor evaluation using the Flywalk paradigm. The contribution of OSN types to odor evaluation has so far been tested in several studies, each concerned with single OSN types using a wide variety of different behavioral paradigms (Ai et al., 2010; Dweck et al., 2013, 2015a,b; Grosjean et al., 2011; Kurtovic et al., 2007; Min et al., 2013; Ronderos et al., 2014; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004). Also, a correlation between the activities of different projection neuron (PN) types and behavior to a large odor set has been established previously (Knaden et al., 2012). However, in order to establish causality rather than correlation, it is necessary to show that OSN output is necessary and sufficient to cause the observed behavioral effect. Therefore, the ability to silence OSN populations is essential to establish a causal relationship between OSN input and behavioral output. Because the behavioral effect of a loss of an individual OSN type can be rather cryptic and may not necessarily strongly affect responses towards the OSN's presumed cognate ligand (Keller and Vosshall, 2007), large-scale approaches will be needed to crack the olfactory valence code, and these depend on dependable genetic tools as it may not be possible to control for efficient silencing in every case.

Our data demonstrate that, at least when expressed heterozygously, none of the tested genetic silencing tools really silenced all OSNs in the literal sense of the word. That being said, the effectors clearly differed in their potencies, with *TeTx* being the most efficient, followed by *Kir2.1*, both of which significantly (*TeTx*) or at least by trend (*Kir2.1*) abolished odor-guided behavior in the trap assay and to some extent also in the open-field arena, and in the Flywalk paradigm when expressed homozygously. In contrast, expression of *DTA* and *rpr* did not affect odor-guided behavior in any of the bioassays. A similar ranking of the potencies of the tested silencers in the motor system has also been reported previously by others (Thum et al., 2006). Therefore, we conclude that these differences are at least in part intrinsic to the effectors, although target cell type and timing of expression may also contribute to the effectiveness.

What may be the mechanistic reason for the observed differences? Both *DTA* and *rpr* act by ultimately killing their target cell. Whereas the action of *rpr* depends on the cellular apoptosis machinery and effectiveness of silencing may therefore vary depending on cell type, *DTA* is an inhibitor of protein synthesis and should therefore be ultimately lethal for all cell types. However, our results suggest incomplete ablation of the *Orco*-expressing OSN population for both *rpr* and *DTA*. Because we used rather high odor concentrations throughout the study, it is conceivable that a low number of surviving OSNs may be sufficient to evoke the behavior. The inefficiency of *DTA* is nevertheless surprising given its extreme toxicity. However, as a protein synthesis inhibitor, its action depends on cellular protein turnover rates and its effect may therefore be observable in flies older than those we tested.

In contrast to *DTA* and *rpr*, *TeTx* worked well in trap assays and both *TeTx* and *Kir2.1* in Flywalk if homozygously expressed. Furthermore, a heterozygous expression of *TeTx* was sufficient to abolish behavior in the open-field arena. Because expressing either *Kir2.1* or *TeTx* heterozygously did not phenocopy responses of *Orco* mutant flies in the Flywalk assay, silencing of OSN output is presumably also incomplete when using these constructs. Incomplete silencing was recently reported for the temperature-sensitive *dynamin* variant *shibire^{ts}*, which is also widely used in *D. melanogaster* behavioral studies and considered to be very effective. In that study, the authors showed that the expression of *shibire^{ts}* in OSNs reduced the responses in postsynaptic PNs by approximately 50% at the restrictive temperature (Liu and Wilson, 2013). A similar incomplete silencing of OSN activity may be a reason for the remaining responses in flies heterozygously expressing *Kir2.1* and *TeTx* in Flywalk, particularly because at least for some of the odors the concentrations we used were well above the behavioral threshold (Thoma et al., 2014).

But why do some constructs abolish behavior in some but not in other bioassays? We assume that the reason for the dependence on the type of bioassay lies in the navigational strategy employed to approach the odor source. In anemotactic assays such as Flywalk, the sole demand on the olfactory system is to identify and evaluate odors, whereas directional cues concerning the location of the odor source are provided by the wind direction. In chemotactic assays such as the trap assay or the open-field arena, odor source localization also depends on the olfactory system, in addition to odor identification and evaluation. *Drosophila melanogaster* larvae evaluate the direction of an odor gradient by an active sampling process, and respond behaviorally to small local concentration increments (Gomez-Marin et al., 2011; Louis et al., 2008). Adult vinegar flies have been demonstrated to be able to measure and respond to local concentration differences across their antennae in tethered paradigms (Borst and Heisenberg, 1982; Gaudry et al., 2013), although it is not entirely clear whether the slope of a natural odor gradient would be sufficiently steep to assess its direction by comparing the difference in inputs to the two antennae. Irrespective of whether adult flies assess the direction of odor gradients by comparing concentration across two spatially separated sensors, or by moving the sensors through the gradient and comparing concentration differences in time, both strategies probably depend on the full dynamic range and contrast of the olfactory system, both because local concentration increments may be tiny and because

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they need to be measured under varying background conditions. Although *TeTx* and *Kir2.1* may not fully silence OSN output, they are likely to reduce dynamic range and contrast of the olfactory system already in the heterozygous state, and this reduction may be sufficient to disrupt chemotactic navigation, but insufficient to disrupt odor evaluation. This may be why the genetic manipulations had a stronger impact on fly behavior in trap assays and the open-field arena than in the Flywalk paradigm.

The observation that flies expressing *TeTx* in OSNs but not *Orco* mutant flies fail to locate the odor source in the open-field arena is rather puzzling and we can only speculate about possible reasons. *TeTx*-expressing flies clearly do not have motor deficits, which could have explained the results, because they also display odor responses in Flywalk (Fig. 2H). The effect of *TeTx* expression is unlikely to be an effect of the genetic background, because both parental strains were attracted by the odor source (Fig. S1D). Both *Orco* mutants and *TeTx*-expressing flies are likely to perceive balsamic vinegar, probably via IR-dependent detection of acetic acid, because both genotypes display attraction towards vinegar in Flywalk (Fig. 2D,F). Hence, our observations suggest that *Orco* mutants rely more on their IR nose during chemotactic close-range search behavior than do flies expressing *TeTx*. The *Orco* mutant strain we used has been published more than 10 years and – assuming an average generation time of 2 weeks – approximately 300 fly generations ago (Larsson et al., 2004). In contrast, the *TeTx*-expressing flies we tested were from the first generation with strongly reduced input from the OR nose. Considering that only 15 generations of experimental evolution are sufficient to induce a significant difference in learning abilities (Mery and Kawecki, 2002) and 30 generations of monogamy are sufficient to significantly reduce female fecundity (Innocenti et al., 2014), it is conceivable that 300 generations without an OR nose may have favored an altered usage and evaluation of the olfactory input from the IR-dependent olfactory system, although the selection pressure is probably low under standard laboratory culture conditions. This is of course highly speculative, but at the same time it appears to be the most parsimonious explanation for our observations and may provide an interesting future avenue of research in the evolution of odor-processing systems.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceived study and designed experiments: T.R., M.T., M.K. and B.S.H. Performed experiments: T.R. Analyzed data: T.R. and M.T. Wrote and revised manuscript: T.R., M.T., M.K. and B.S.H.

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Supplementary information
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Manuscript II

Adult Frass Provides a Pheromone Signature for *Drosophila* Feeding and Aggregation

Ian W. Keesey, Sarah Koerte, Tom Retzke, Alexander Haverkamp, Bill S. Hansson, Markus Knaden

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Adult Frass Provides a Pheromone Signature for *Drosophila* Feeding and Aggregation

Ian W. Keesey[1] · Sarah Koerte[1] · Tom Retzke[1] · Alexander Haverkamp[1] · Bill S. Hansson[1] · Markus Knaden[1]

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Abstract Adult *Drosophila melanogaster* locate food resources by using distinct olfactory cues that often are associated with the fermentation of fruit. However, in addition to being an odorous food source and providing a possible site for oviposition, fermenting fruit also provides a physical substrate upon which flies can attract and court a potential mate. In this study, we demonstrate that *Drosophila* adults are able to recruit additional flies to a food source by covering the exposed surface area with fecal spots, and that this recruitment is mediated via olfactory receptors (Ors). Analyses of the deposited frass material demonstrates that frass contains several previously studied pheromone components, such as methyl laurate (ML), methyl myristate (MM), methyl palmitate (MP), and 11-*cis*-vaccenyl acetate (cVA), in addition to several cuticular hydrocarbons (CHCs) that are known to be behaviorally active. Moreover, this study also demonstrates that adult feeding is increased in the presence of frass, although it appears that Ors are less likely to mediate this phenomenon. In summary, the frass deposited by the fly onto the fruit provides both pheromone and CHC cues that lead to increased feeding and aggregation in *Drosophila*. This research is the first step in examining *Drosophila* frass as an important chemical signature that provides information about both the sex and the species of the fly that generated the fecal spots.

Keywords Olfactory · Gustatory · Chemical ecology · *Drosophila* · Frass · Feces · Pheromones · Insect behavior

Hansson and Knaden shared seniority and last authorship

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✉ Bill S. Hansson
hansson@ice.mpg.de

✉ Markus Knaden
mknaden@ice.mpg.de

[1] Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Jena, Germany

Introduction

The pheromone system of *Drosophila* has been extensively studied, and previous research provides detailed information on the chemical identity of behaviorally relevant compounds that are generated by male and female flies (Auer and Benton 2016). This broad area of research also delves deeply into the neuronal mechanisms for both the detection and the decision-making of the fly in response to the presence of these pheromones, including the governance of complex multi-modal phenomena such as mate recognition and courtship. Recently, several important olfactory receptor ligands were uncovered, including methyl laurate (ML), methyl myristate (MM), and methyl palmitate (MP), which are some of the best known ligands for pheromone receptors Or47b and Or88a (Dweck et al. 2015). In addition, work by Lin et al. (2016) also suggests that myristic acid, palmitoleic acid, and palmitic acid could also act as important ligands as well. These two new studies provide olfactory ligands that fit nicely into the already established model for the neuronal activation of these circuits; however, the origin and production site of these fatty acid derived ligands has not yet been determined.

Feces collected from various insects has been previously studied for several attributes such as chemistry, shape, and color (Kuhns et al. 2012; Shao et al. 2012; Tumlinson et al. 1969; Wayland et al. 2014). In the case of the boll weevil, the examination of frass provided the behavioral relevance and eventually the identification of specific pheromone components that were

otherwise difficult to isolate from adult odor collections or from the associated chemical analyses of courtship (Tumlinson et al. 1969). More recently the importance of fecal pheromones in aggregation behavior also was demonstrated in the German cockroach, *Blattella germanica*, where researchers showed that this insect emits highly attractive carboxylic acids in healthy adult feces (Wada-Katsumata et al. 2015). It also has been noted that frass can provide behaviorally relevant cues to parasitoids, such as wasps that target larvae of the diamondback moth (Reddy et al. 2002). Thus, frass across the order Insecta already has been established as a well-known substrate for behaviorally relevant odor cues.

Previous examination of *Drosophila melanogaster* frass has yielded information concerning the physical properties such as shape, size, and optical density of fecal droplets. These studies provided interesting differences in frass that depend on mating status and sex of each *D. melanogaster* fly that was tested (Wayland et al. 2014). In addition, researchers also have examined frass in regard to the quantification of fecal production, as well as the concentration of fecal material, in order to generate data on total excretion and water reabsorption (Linford et al. 2015; Urquhat-Cronish and Sokolowski 2014; Wayland et al. 2014). These studies showed the importance of frass in non-invasive studies of *Drosophila* metabolism and suggested that frass could be used as a metric for assessing general health, especially as it pertains to either nutrient or microbial stress. However, no previous studies have examined *Drosophila* frass in regard to its chemical properties or tested this digestive byproduct for any behavioral relevance. Here, we first document strong attraction of *Drosophila* adults towards frass, as well as demonstrate the presence of several CHCs and pheromones. We also provide a protocol for the collection of fecal material, as well as potential procedures for the examination of sex- and species-specific differences between fecal collections across this genus of flies.

Materials and Methods

Fly Stocks All wildtype fly lines, including *D. simulans* (14,021–0251.195), *D.erecta* (14,021–0224.01), *D. mauritiana* (14,021–0224.01), *D. virilis* (15,010–1051.00), *D. suzukii* (14,023–0311.01), *D. biarmipes* (14,023–0361.10), and *D. pseudoobscura* (14,021–0121.94) were obtained from the UCSD Drosophila Stock Center (www.stockcenter.ucsd.edu). All experiments with wild-type *D. melanogaster* were carried out with Canton-S (WTcs, stock #1), which were obtained from the Bloomington *Drosophila* Stock Center (www.flystocks.bio.indiana.edu). Stocks were maintained according to previous studies, and for all behavioral experiments we used 2–5 d-old flies of both sexes.

Stimuli and Chemical Analysis All of the synthetic odorants that were tested and confirmed were acquired from commercial sources (Sigma, www.sigmaaldrich.com and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery for behavioral experiments followed previously established procedures, and any headspace collection of volatile odors was carried out according to standard procedures (Keesey et al. 2015). Blueberries were selectively used for fruit experiments since *D. melanogaster* could not penetrate or oviposit through the hardened surface of the berries. In addition, the small size of the blueberry allowed the use of intact, completely sealed fruit, which further prevented *D. melanogaster* from gaining any access beneath the surface or skin of the berry. GC-MS analyses were performed on all volatile and insect body wash collections as described previously (Dweck et al. 2015). The NIST mass-spectral library identifications were confirmed with chemical standards where available.

Frass Collections The sides of rearing vials that contained 100 adult flies were scraped after 1 wk. with a flat, rounded-end micro spatula. Each rearing vial could be separated into distinct zones of pupation as well as frass deposition (Supplemental 3), and thus no larvae or pupal cases were included in these frass collections. After scraping was completed, 150–200 mg of frass were added to either 1 ml of water, methanol, or hexane solvent. After 24 h, collected material was filtered through sterilized paper disks to remove large particles, and then these frass infused solvents were used in behavioral trials with the addition of mineral oil.

Behavioral Assays Trap assays were performed with 2–5 d-old flies as previously described (Keesey et al. 2015; Knaden et al. 2012), but with an additional 200 μl of light mineral oil (Sigma-Aldrich, 330,779–1 L) that was added to capture and drown flies upon contact with the treatment or control within the container. All behavioral traps consisted of 60 ml plastic containers (Rotilabo sterile screw cap, Carl Roth GmbH, EA77.1), with one trap used as a solvent control and the other containing the treatment (Fig. 3f). All trap experiments were repeated using water, methanol, or hexane as solvents for the frass collections. While all solvents generated significant attraction towards frass when compared to the control, water was the best solvent for behavior, but it could not be used for further GC-MS analyses, thus methanol was utilized instead for all additional experiments with *Drosophila* frass, as it had the closest polarity to water. Flywalk trials also were conducted as described previously (Steck et al. 2012; Thoma et al. 2014; Supplemental Fig. 5). In short, 15 flies were placed individually into parallel glass tubes. During the experiment, flies were exposed continuously to a humidified airflow of 20 cm/s (70 % relative humidity, 20 °C). Flies were presented repeatedly with air pulses from the head space of frass solved

in water, or to pulses of water alone, at an interstimulus interval of 90 s for 8 h. The 500 ms pulsed air stimuli were added to the continuous airstream and thus traveled through the glass tubes at a constant speed. The individual flies' movements before and after stimulus arrival were monitored under red-light conditions using advanced video-tracking software (Steck et al. 2012; Thoma et al. 2014).

Feeding Assays All tested flies were 2–5-d-old, included both males and females, and were starved beforehand for 18–20 h with constant access to water. Flies then were cooled for 5 min at -20 °C to assist in their transfer to the petri dish arena. Basic feeding solutions consisted of water with 5 % sucrose and 5 % baker's yeast, and experiments were conducted with or without colored dye markers (red and blue). Frass was added to treatment solutions, and included 150–200 mg of material per 1 ml of sugar water. After the 20 flies entered the arena, observations of fly feeding behavior were made at 2 min intervals for 30 min. Flies that fed on dye markers then were frozen at -20 °C, and images were taken for counting and additional analyses. The capillary feeder (CAFÉ) assays utilized glass micropipettes with liquid media that were filled by capillary action, and then inserted through pipette tips into the container holding the adult flies (modified from Ja et al. 2007). One capillary contained the control (5 % sucrose), while the other contained the treatment (5 % sucrose plus frass), and the volume consumed from each side was measured after a set duration of fly feeding.

Results

Fecal Deposits on Fruit *Drosophila* adults that had access to fruits, deposited fecal spots directly onto the fruit surface area using randomly spaced, often non-overlapping droplets (Fig. 1a, b). Surface washings of the fruit with and without deposited fecal spots, and solvent extractions of frass material alone revealed that several behaviorally important compounds were present in association with these fecal droplets, including the recently described pheromone components methyl laurate (ML), methyl myristate (MM), and methyl palmitate (MP), as well as their corresponding acids (lauric acid, myristic acid, palmitoleic acid, and palmitic acid). In a trap assay, when *Drosophila* adults were allowed to choose between the odor of fruit alone, and the odor of fruit that had been in contact with other *Drosophila*, the majority of flies selected the fruit with previous exposure to conspecifics (Fig. 1c). To ascertain the chemical profile of the frass alone, the fecal deposits were collected along the sides of the clear plastic rearing vials and placed into three solvents, which included water, methanol and hexane (Fig. 1d; Supplemental Fig. 3). Although water and methanol extracts were the most consistently attractive, all three fecal solvent extractions produced attraction in WT flies (i.e., wildtype flies of the Canton S strain) and w1118 control flies (i.e., white eye flies that carry the same genetic background as the other tested mutant fly lines). It also was noted that water completely dissolved the fecal material while hexane did not, suggesting that the frass contains predominantly polar compounds.

Differences between Male and Female Frass To test for any differences between male and female frass, newly emerged virgin flies were collected and placed into separate rearing vials based on sex. Subsequent fecal collection was completed as described previously (Supplemental Fig. 3), and this sex-specific frass material was added to methanol for further chemical analyses. By comparing adult body washes to these sex-specific fecal profiles by using GC-MS, it was demonstrated that frass contains information regarding the sex of the fly (Fig. 1d; Supplemental 6 A, B), and moreover, that the chemical signature of the frass matches most closely the *Drosophila* adult that produced it (Fig. 1d). More specifically, the GC-MS data showed that feces of both sexes contain the recently described pheromones ML, MM, and MP, while male feces contains a large amount of 11-*cis*-vaccenyl acetate (cVA) and 7-tricosene (7 T), and that female feces contains higher amounts of (7Z-11Z)-heptacosadiene (7,11-HD) and (7Z,11Z)-nonacosadiene (7,11-ND), which matches previously reported adult pheromone and adult CHC profile differences between the two sexes (Auer and Benton 2016; Dweck et al. 2015).

Attraction Towards Frass To test the behavioral relevance of frass, trap assays were used to compare the solvent control against the fecal collections. For water, methanol and hexane solvents, the frass was significantly more attractive than the evaporated solvent controls (Fig. 2a; WT, Canton S and w1118, white eyes; methanol data shown). Next, to examine the importance of odorant receptors, mutant flies lacking a functional olfactory co-receptor (Orco) were tested for their attraction towards frass. These mutant flies displayed a significantly reduced but still significant behavioral preference for frass, suggesting that at least part of the attraction towards frass was mediated by olfactory sensory neurons expressing odorant receptors, but also that other types of receptors were involved. To further address the importance of previously identified pheromone components in the attraction towards frass, multiple mutant fly lines were utilized that were only deficient in specific pheromone receptors, including Or47b (detecting ML), Or67d (detecting cVA), and Or88a (detecting ML, MM, and MP). All three of these mutant fly lines demonstrated reduced attraction towards frass, and all three were significantly different from the two control fly lines (WTcs and w1118); moreover, these mutant fly lines were not statistically different from the ORCO mutant line, further suggesting the important role of olfactory pheromone receptors in the

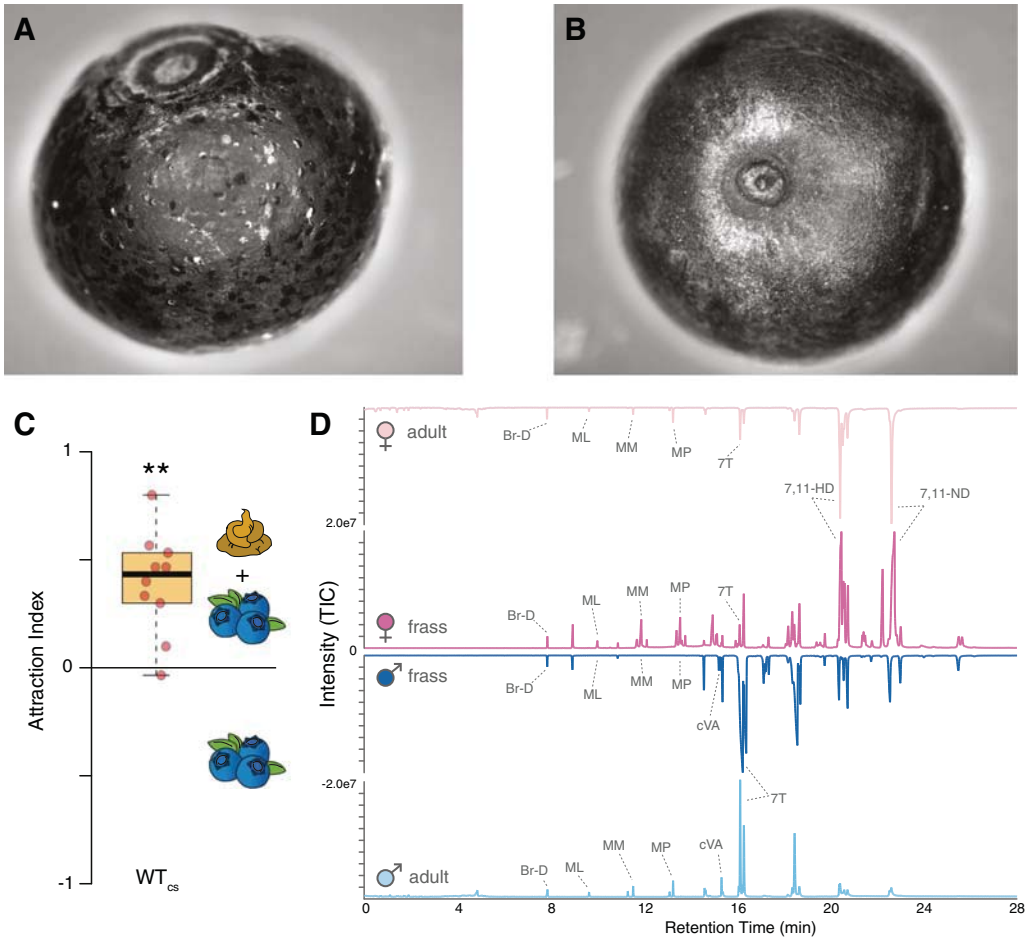

Fig. 1 **a** Image of a blueberry that was exposed to *Drosophila melanogaster* flies for 24 h, where the flies randomly distribute droplets of feces to cover the entire exposed surface area of the fruit. **b** Blueberry without exposure to flies. **c** Trap assays using fruit with and without previous fly contact (i.e., with and without fecal spots), where the fruit with *Drosophila* frass was preferred over the fruit alone. Attraction indices were calculated as (O-C)/T, where O is the number of flies observed in the treatment trap, C is the number of flies in the control trap, and T is the total number of flies used in the trial. **d** Adult male and female chemical profiles were established via short body washes in solvent, and the same procedures were used for GC-MS analyses of frass. Both male and female frass contained significant amounts of previously identified pheromone components, and each frass sample most closely resembles the sex of the adult that produced it. (Br-D, bromodecane [internal standard]; ML, methyl laurate; MM, methyl myristate; MP, methyl palmitate; 7 T, (Z)-7-tricosene; cVA, cis-vaccenyl acetate; 7,11-HD, (7Z, 11Z)-heptacosadiene; 7,11-ND, (7Z, 11Z)-nonacosadiene)

behavioral attraction of adult flies towards frass material (Fig. 2a). To test that all mutant lines (Or47b, Or67d, Or88a) were still behaviorally functional, additional trap assays were conducted with vinegar, which is a general attractant that does not rely on pheromone receptors for attraction (Fig. 2b). While Orco mutant flies were still deficient in their attraction towards vinegar, the three pheromone receptor mutants (Or47b, Or67d, Or88a) all displayed the same level of attraction to vinegar as both control lines, suggesting that these mutant flies exhibited normal behavior towards attractants that do not rely on pheromone detection. Therefore we conclude that the reduced response to frass by these three pheromone mutant lines is due to their loss of specific pheromone Ors. To further test the role of frass in aggregation and attraction, the Flywalk was utilized as well (Thoma et al. 2014; Supplemental Fig. 5D). Using this behavioral paradigm it was demonstrated that the odor of frass was indeed more attractive than the water control for both virgin and mated males ($P < 0.01$), as well as for both virgin and mated females ($P < 0.01$) (Fig. 2c), with flies reaching walking speeds towards frass odor that exceeded those previously published with some of the best *Drosophila* attractants such as ethyl acetate and ethyl butyrate (Thoma et al. 2014). There was no significant difference between mated and virgin males ($P > 0.05$), nor was there any significant difference between mated and virgin females ($P > 0.05$). However, mated males were significantly more attracted than

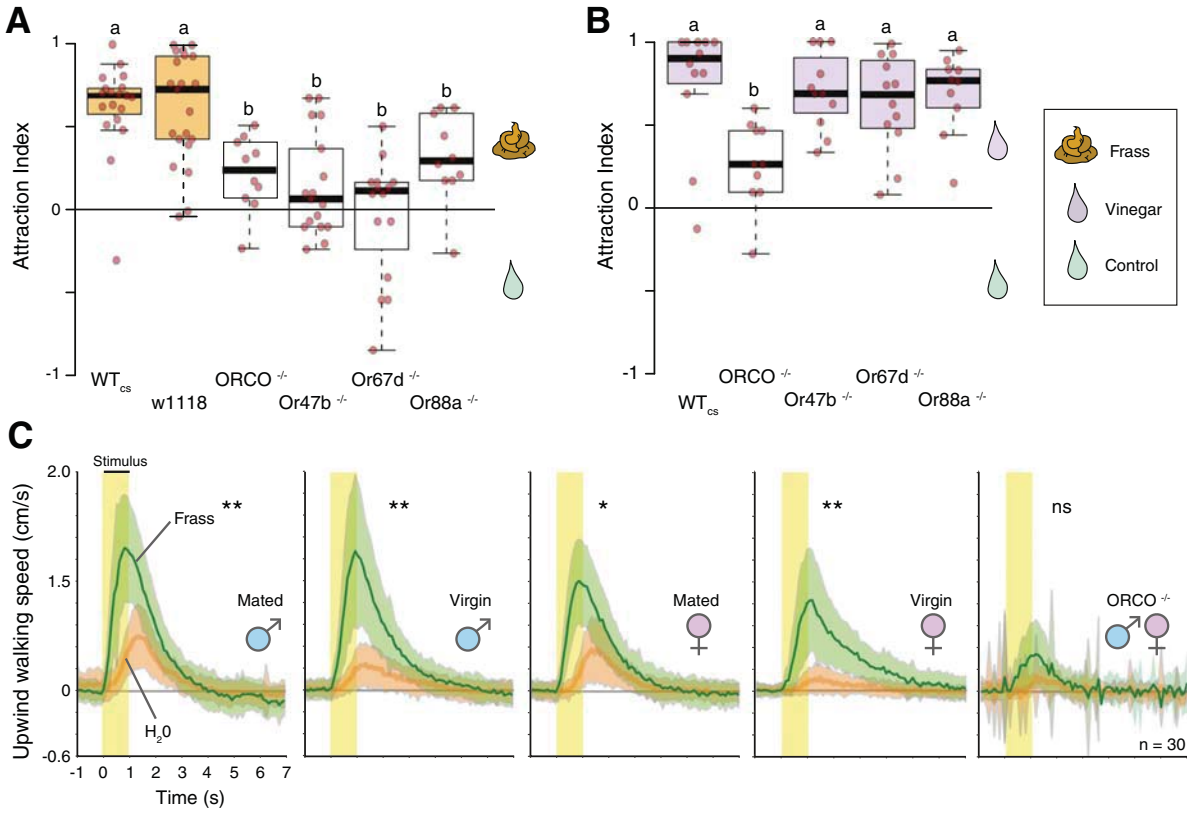

Fig. 2 Attraction indices from trap assays containing either **a** frass or **b** vinegar. Data includes flies deficient in either Orco or pheromone-specific Ors, and also shown are the corresponding responses of wild type (WTcs, *Drosophila melanogaster* Canton S) and other transgenic control flies (w1118, white eye). Attraction indices were calculated as (O-C)/T, where O is the number of flies observed in the treatment trap, C is the number of flies in the control trap, and T is the total number of flies used in the trial. **c** Responses to frass vs. the water control in the Flywalk, which includes behavioral response data from mated and virgin, as well as male and female adults. Both males and females are significantly attracted towards frass at all time intervals ($P < 0.01$). Males were significantly more attracted than females, regardless of mating status ($P < 0.01$). Tests with Orco flies did not produce any significant attraction towards frass

mated females towards frass ($P < 0.01$), and virgin males were more attracted than virgin females ($P < 0.01$). As was shown with the previously reported trap assays, the Orco mutant line again was significantly less attracted to frass than either WT males or females (Fig. 3c). In addition, behavioral trials were conducted with either virgin female or virgin male frass vs. a solvent control, and each trial produced statistically identical attraction, with both male and female frass being behaviorally attractive in trap assays (Supplemental 6C). In summary, the data show that frass is a strong attractant across several tested behavioral paradigms for *Drosophila* attraction and aggregation, and that both male and female frass is attractive.

The Effect of Frass on Feeding Behavior We conducted three sets of feeding trials, first using food dye to determine the preference of *D. melanogaster* for feeding on substrates infused with frass (Fig. 3a). Regardless of whether red or blue dye was used, flies preferred to feed from solutions containing frass (Fig. 3a; Supplemental Fig. 4). To confirm that flies were feeding in addition to aggregating at the solution, images of the colored dye were taken after the feeding trials were completed (Supplemental Fig. 4). In a second feeding trial, in this case without dye and during 30 min of direct observation with starved flies, the feeding solution containing frass again was significantly preferred over the control solution (Fig. 3b). In addition, we conducted a third set of feeding trials using CAFÉ assays, which compared 5 % sugar water (control) to the same solution with the addition of fecal material (Fig. 3c). In these trials, WT control flies fed more from the treatment containing frass; however, we also observed that ORCO flies preferred to feed from the capillary that contained frass (Fig. 3c), suggesting that while feeding is enhanced by fecal material, that this increase is perhaps not directly influenced by odorant receptors.

Examination of Frass from Different Species Having shown that frass from *D. melanogaster* contains a sex-specific

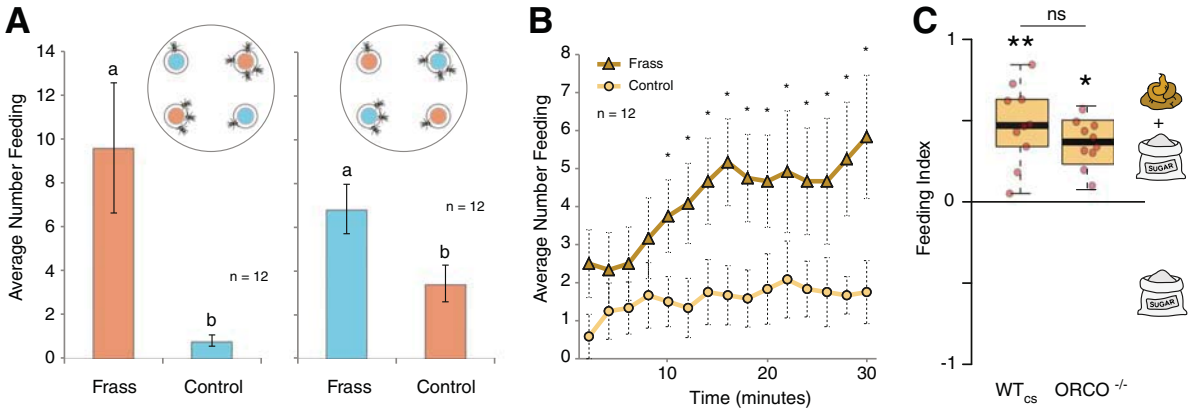

Fig. 3 Assays comparing feeding on sugar solution alone vs. sugar solution that also contain frass. Trials were conducted with both red and blue dye. Feeding behavior of each fly was documented based on the amount of red or blue dye in the abdomen after a 30 min exposure to the food (see also Supplementary Fig. 4). **b** Numbers of flies that were observed feeding at the frass-containing and the control food sources during 2 min intervals of direct observation for a total of 30 min. Flies contacted and fed upon frass-containing sugar solutions significantly more than the controls. **c** Feeding indices of wildtype and Orco mutant flies using a CAFÉ assay with 5 % sucrose solution either with or without frass. Significant differences are denoted by letters or asterisks (ANOVA followed by Tukey's test; $P < 0.05$). Error bars represent SEM

combination of CHCs and pheromones, our next interest was determining whether different *Drosophila* species contained notable differences in their frass. To test this we examined eight species of *Drosophila* flies, and compared the male and female adult body washes of each species to their corresponding fecal collections. We examined GC-MS data from 600 s onward, which included a total of 69 distinct compounds across the 8 fly species, and the data were normalized to the total amount of peak area in each total ion chromatogram (TIC). Data were log transformed to ensure normality, which was checked by the Shapiro-Wilk test. We used open-source XCMS implemented into the statistical program R to align the raw total ion traces (Smith et al. 2006), which we then used for the PCA, with PCA1 explaining 28 % and PCA2 explaining 16 % of the total variance. In the case of the *melanogaster* clade, all species that we examined produced remarkably similar chemical profiles, not just in the adult body washes, but also in their frass (Fig. 4a; Supplemental 1, 2). While the *melanogaster* relatives (*D. erecta, D. mauritiana, D. simulans*) all produced similar levels of ML, MM, and MP in their frass to that of *D. melanogaster*, there were small differences regarding both cVA content as well as other specific CHCs.

When our analyses was expanded to include more distant relatives of the family Drosophilidae, we were able to demonstrate species-specific differences in fecal deposits (Fig. 4a) in addition to the differences that were observed between adult males and adult females of each species (Fig. 4a; Supplemental 1, 2). Thus, frass appears to provide a chemical signature for each species, and provides species-specific markers to identify as well as leave behind information about the flies that were previously present. In general, the frass that was generated appeared to mirror the adult CHC and pheromone profile. While all examined species and their frass contain pheromone components such as ML, MM, and MP, many species and their corresponding frass appears to be deficient in cVA, further confirming that this compound and other male-produced compounds may be more indicative of species differences than other behaviorally relevant odors. For example, we were able only to identify a minuscule amount of cVA that was generated by *D. suzukii* or *D. virilis*, which had been suggested previously (Dekker et al. 2015), but other species such as *D. biarmipes* appeared to contain larger amounts of this pheromone component in adult male male body washes as well as in collected male frass.

Attraction of Frass from Different Species To test for behavioral differences between the frass collected from different *Drosophila* species, we again utilized the Flywalk. Here we tested the response of *D. melanogaster* adult males towards odor pulses from the frass collected from several different species. While *D. melanogaster* adults were equally attracted to 45 mg of frass from closely-related species (*D. melanogaster*, *D. mauritiana, D. simulans, and D. erecta*), they were significantly less attracted to the odor pulses from more distantly related fly species such as the fecal collections from *D. virilis* (Fig. 4b).

Discussion

In this study, we showed that *Drosophila* frass is behaviorally attractive, and that it provides chemical cues for aggregation in *Drosophila*. Our data also demonstrate that this attraction is predominantly due to the presence of pheromone compounds within the fecal droplets, specifically, the ligands that activate Or47b, Or88a, and Or67d (ML, MM, MP, and cVA,

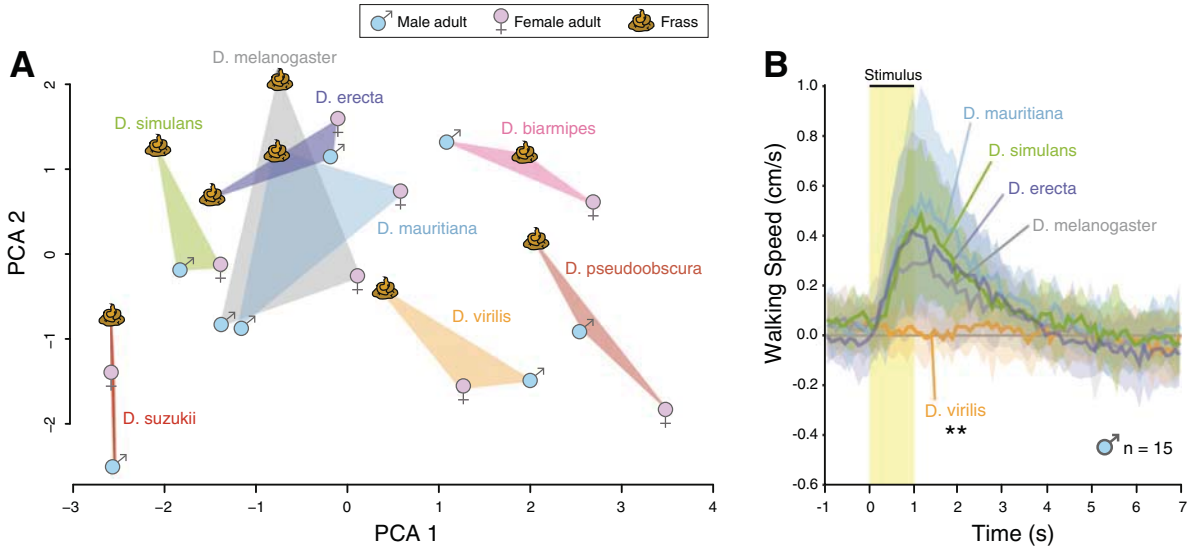

Fig. 4 **a** PCA (variance–covariance matrix) of normalized and quantified major peaks within the GC-MS profiles for 8 species of *Drosophila* flies, including adult male, adult female, and adult frass collections. Several species differ significantly from each other (one-way ANOSIM; Bray–Curtis distance; $R = 0.78$; $P < 0.001$), with the melanogaster clade clustering together without significant differences (*D. simulans*, *D. melanogaster*, *D. erecta,* and *D. mauritiana*; $P > 0.05$). The frass samples collected from *D. suzukii*, *D. biarmipes*, *D. pseudoobscura,* and *D. virilis* were all significantly different from each other, and from the *D. melanogaster* clade ($P < 0.05$). **b** Behavioral trials using *D. melanogaster* adults in the Flywalk that were given the choice between frass collected from several different *Drosophila* species. Flies showed no difference in attraction for closely-related species within the same clade, but were not attracted to the frass from more distant relatives such as *D. virilis*

respectively). Moreover, the importance of MM, ML, and MP and their role in aggregation and courtship already has been demonstrated (Dweck et al. 2015). Recent work by Lin et al. (2016) has suggested that several fatty acids (i.e., myristic acid, palmitoleic acid, and palmitic acid) also strongly activate Or47b, and our analyses has shown that these compounds are also all found in high abundance in the frass. It also has been previously established that 7-T and 9-T inhibit mating between species and contribute to aggregation (Fan et al. 2013), and our current study confirmed that these CHC compounds were found in high abundance within the fecal droplets as well. Numerous studies have shown that cVA has roles in aggregation, in mating deterrence, in male-male aggression, and that this compound is passed from males to females as an anti-aphrodisiac during mating (Auer and Benton 2016). Given all this information, our data suggest that frass also could achieve these same behavioral outcomes through the activation of the same neuronal circuits, due to the presence of the before mentioned chemistry (ML, MM, MP, and cVA, as well as their corresponding acids), and thus that frass is to a great extent a general aggregation signal that is composed of robust gustatory and olfactory cues. However, future work is necessary to examine the importance of frass in other *Drosophila* behaviors beyond attraction, such as mate recognition, courtship, male-male aggression, and oviposition.

In subsequent experiments we also generated evidence that the presence of frass increases feeding behavior. Given that this increase in feeding appears to not be mediated by olfactory receptors, as demonstrated by the use of Orco mutants (Fig. 3c), future studies will target the possible role of gustatory (Gr), as well as ionotropic (Ir) and PPK receptors. Since 7-T is detected by gustatory neurons expressing Gr32a (Wang et al. 2011), this receptor might be a candidate in mediating the increased feeding. It also is worth noting that while the contents of *Drosophila* frass have not yet been analyzed specifically for microorganisms, it is likely that this fecal material contains both yeast and bacteria in addition to the described pheromone components. It recently has been shown that specific Grs and Irs are responsible for the increased feeding and mating receptivity afforded by the presence of yeast (Gorter et al. 2016). Therefore, the increased feeding on solutions containing frass is most likely at least partially linked to these same taste receptors, although more work is needed to test this hypothesis, and to further examine the presence of potential microorganisms in *Drosophila* frass.

The frass collected from each sex and each species of fly appears to match the odor profile of the adult that produced it (Fig. 4a). This similarity between adult and frass chemistry is not surprising given that the alimentary canal consists of a cuticular material similar to that which forms the outer epi- and exocuticle. It is thus reasonable that frass content positively correlates to the exterior pheromone and CHC profile of the adult fly (Fig. 4a). The data reported here support the current literature that *Drosophila* can discriminate between species-

related chemical differences among adults, but our data go one step further and also support the notion that *Drosophila* can discriminate between the frass or fecal deposits left behind by distantly related species at a food source (Fig. 4a, b). While it has not been shown previously that frass from *Drosophila* contains behaviorally relevant chemical stimuli, this has been demonstrated repeatedly for other insect orders, including Coleoptera and Blattodea (Symonds and Gitau-Clarke 2016; Wada-Katsumata et al. 2015). In research with other insects, frass has also been shown to provide a substrate that can be used to identify novel pheromone components from several agricultural and economic pests, such as the boll weevil and the many destructive species of pine beetle (Bellas et al. 1969; Hall et al. 2002; Symonds and Gitau-Clarke 2016; Tumlinson et al. 1969).

While previous work has identified the presence of pheromones as part of the fecal signature in these insects, it has not been shown that *Drosophila* frass also contains sex-specific and species-specific markers. Therefore, our current investigation of frass chemistry provides several avenues for future application, such as the identification of novel pheromone components from additional insect species, especially in cases where the induction of calling behaviors or where the release of pheromones is difficult to stimulate in the laboratory. Examination of *Drosophila* frass also provides novel approaches to the studies of economically important species within this genus, such as *D. suzukii*, where the loss of cVA might have been replaced by another behaviorally relevant male-generated pheromone component that could be more easily identified from fecal studies. It also is likely that certain chemical components of *D. suzukii* frass could provide species-specific attraction and aggregation cues that in turn may benefit current IPM strategies.

While frass from otherwise healthy adults is behaviorally attractive, it is not yet determined whether diet or other external influences can modify the chemical signature of feces. It would be interesting to address whether the chemistry of frass changes in regard to food resources, such as in *Drosophila* reared upon different food substrates (e.g., food deficient in amino acids or sugars) or by rearing the flies upon the same fruit at different stages of decay. Moreover, it would be interesting to ascertain whether the frass itself changes after exposure to or ingestion of different healthy or pathogenic microbes that have been incorporated into the diet, such as different yeast or bacteria strains. It is possible that frass can provide a signature or snapshot of individual insect health, or perhaps insect population health, especially as it relates to mid- and hindgut metabolism (Kuhns et al. 2012; Newell and Douglas 2014). Additional work is also required to ascertain whether the frass itself affects the substrate that it is deposited onto, namely the fruit or food resource utilized by each *Drosophila* species. While it is clear that frass contains pheromone components, and that frass is involved in the attraction or recruitment of other *Drosophila* to a food source, it still is open for debate whether the frass itself is an active substance that plays any role in breaking down food resources, such as through the utilization of gut microbes, including yeasts or bacteria, or through the use of enzymatic and digestive substances that are potentially deposited along with or within the fecal spots. In the present study, we showed that flies deposit frass in a rather random, but often non-overlapping distribution across the entire exposed surface area of potential food substrates (Fig. 1a). Therefore frass may aid in the decay or fermentation of nutrient resources through the recruitment or deposition of microorganisms. It has already been demonstrated that ingested microbes such as yeast spores can survive the digestive tract of *Drosophila* (Coluccio et al. 2008; Erkosar and Leulier 2014). Thus, it is likely that different species of *Drosophila* produce frass that contains different strains of microorganisms that could in turn be distributed through fecal spots to assist or accelerate the breakdown of species-specific food resources (e.g., cacti, mushrooms, or fruit) (Wong et al. 2013, 2014). This scenario would potentially benefit both the fly and the microorganisms that they in turn vector to each new host plant.

It is clear from the present study that frass contains relevant chemical information for each *Drosophila* species and that fecal deposits appear to play a role in both feeding and aggregation. However, it is not yet clear whether frass plays any additional roles in aspects of courtship, or whether frass affects oviposition decisions, such as site selection. It has been demonstrated that some species of flies such as Tephritids leave oviposition marks that ward off other females (Arrendondo and Diaz-Fleischer 2006). Thus, it is possible that some species of *Drosophila* might utilize similar fecal deposits to mark fruit after oviposition, especially in cases when eggs are either laid singly or where they are laid in tight clusters. A recent study has examined sperm plugs containing cVA that are deposited by mated *Drosophila* females that enhance oviposition (Dumenil et al. 2016). Potentially, we could have overlooked sperm plugs when collecting mated female feces for examination. However, as feces from virgin females and virgin males were both significantly attractive to adult flies (Supplementary Fig. 6C), we can conclude that additional cues besides cVA are involved in fly attraction towards frass. Nevertheless, specific studies examining the effects of frass on oviposition also are still required, and future studies will need to separate the contributions of cVA from the other pheromone cues found in frass. Currently, one of the more economically important *Drosophila* species, *D. suzukii*, would be a prime candidate for a more extensive study of frass in regard to attraction, avoidance or oviposition, as any attractive or deterrent chemistry from frass may aid in IPM strategies towards the control of this pest insect. While we were able to show the presence of cVA in *D. suzukii* for both adult extractions and within male frass, albeit greatly reduced compared to

D. melanogaster, it is possible that another male-produced compound is still passed from males to females during copulation in this species, and thus frass material may provide an avenue for the identification of such novel chemistry. In summary, as growing evidence continues to support an intimate association between *Drosophila* and distinct microorganisms, it is clear from our study that additional research should be conducted to examine *Drosophila* frass and its role in the chemical ecology of this genus of fly.

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Manuscript III

Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

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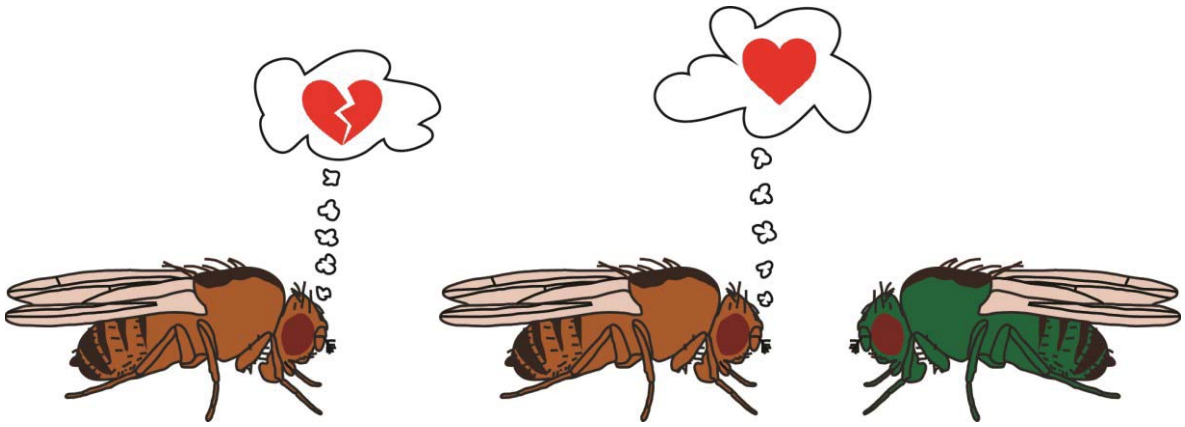

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Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

Ian W. Keesey[1], Sarah Koerte[1], Mohammed A. Khallaf[1], Tom Retzke[1], Aurélien Guillou[2], Ewald Grosse-Wilde[1], Nicolas Buchon[2], Markus Knaden[1] & Bill S. Hansson[1]

Pathogens and parasites can manipulate their hosts to optimize their own fitness. For instance, bacterial pathogens have been shown to affect their host plants' volatile and non-volatile metabolites, which results in increased attraction of insect vectors to the plant, and, hence, to increased pathogen dispersal. Behavioral manipulation by parasites has also been shown for mice, snails and zebrafish as well as for insects. Here we show that infection by pathogenic bacteria alters the social communication system of *Drosophila melanogaster*. More specifically, infected flies and their frass emit dramatically increased amounts of fly odors, including the aggregation pheromones methyl laurate, methyl myristate, and methyl palmitate, attracting healthy flies, which in turn become infected and further enhance pathogen dispersal. Thus, olfactory cues for attraction and aggregation are vulnerable to pathogenic manipulation, and we show that the alteration of social pheromones can be beneficial to the microbe while detrimental to the insect host.

[1] Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Straße 8, D-07745 Jena, Germany. [2] Department of Entomology, Cornell University, 5124 Comstock Hall, Ithaca, NY 14853, USA. Markus Knaden and Bill S. Hansson contributed equally to this work. Correspondence and requests for materials should be addressed to M.K. (email: mknaden@ice.mpg.de) or to B.S.H. (email: hansson@ice.mpg.de)

Certain pathogens, parasites, and viruses possess the ability to manipulate their host, including examples in vertebrates[1–3], invertebrates[4–7] as well as in plants[8–10]. For instance, bacterial pathogens use several strategies to hijack plant host physiology to their own benefit while often to the detriment of their host plant, including alterations of volatile and non-volatile host metabolites and immune-related proteins. This change in volatile release after host–plant infection can also lead to an enhanced attraction of insect vectors to the infected plant, and can therefore lead to increased pathogen dispersal by insect vectors[8, 9, 11]. It has also been shown that a pathogenic bacterium, *Pseudomonas syringae*, is able to alter the physiology of its plant host, *Arabidopsis*, in order to enhance bacterial growth and to help the bacterium avoid defensive responses within the host by altering hormone signaling as well as host susceptibility[10]. In the case of the parasitic flatworm, *Leucochloridium paradoxum*, it infects land snails and the parasite congregates in the eye stalks, where it pulsates different colors and patterns in a display to make the snail more noticeable to bird predators, which are the primary host of this flatworm[6]. Similarly, rats and mice lose their fear of cats upon infection with the parasite *Toxoplasma gondii* and subsequently become more likely to be killed and consumed by a cat, again the primary host of the parasite[12]. This fearless or suicidal behavior in mice has subsequently been shown to be due to an impairment of the olfactory receptors that usually trigger aversion to feline urine, and that this olfactory impairment is caused directly via the infection by the *Toxoplasma* parasite[1, 13]. Other systems for the study of pathogenic alteration of behavior include several examples within insect hosts, such as ants[5, 14], crickets[4], and leafhoppers[11]. Thus, in both plants and animals, microorganisms have been shown to alter the behavior and physiology of a host in order to provide a benefit to the pathogen. However, especially in animal systems, the specific mechanisms for host alteration by pathogens and parasites are not well understood.

Drosophila has been a powerful model to study bacterial infection as it pertains to immune, hormonal, and metabolic responses mounted by the insect host[15–18]. Several strains of pathogenic bacteria, including *Erwinia carotovora* sp. *carotovora* 15 (Ecc15), *Serratia marescens Db11*, and *Pseudomonas entomophila*, have been well characterized in regard to the immune responses elicited by *Drosophila melanogaster* following infection[15, 19–22], and thus these bacteria have arisen as a part of a model system for the study of insect immunity. Although *D. melanogaster* does not possess an adaptive immune system, their innate immune defense has proven to be efficient against most bacteria that are ingested or injected into the fly, perhaps an evolutionary result of living and breeding in high-density, and within microbe-rich food substrates such as rotten and decaying fruit[17, 23]. The *Erwinia* bacterium we use in this study is a member of the Gram-negative Enterobacteriaceae family, several species of which are phytopathogenic, often causing soft rots on fleshy fruits, vegetables, and ornamental crops[24, 25]. This bacterial pathogen has developed sustained plant-to-plant infection cycles, usually via insect vectors such as Hymenopterans and Dipterans[24, 25]. This bacterium also overlaps with the preferred host range of *D. melanogaster*, an insect that has a strong preference for decaying or rotting substrates. Moreover, *D. melanogaster* has been previously shown to be a natural vector for *Erwinia carotovora carotovora* and *E. carotovora atroseptica*, both of which cause potato blackleg disease. *Drosophila* are found naturally carrying these strains of bacteria in potato fields, and, at least under greenhouse conditions, it has been established that the vinegar fly is able to vector blackleg disease between potato plants[26, 27]. Similarly, *P. entomophila* was originally described from field-collected *Drosophila*[20]; thus, fly infection by this bacteria is also thought to be naturally occurring. In addition, the strain of *S. marcescens* we use is highly pathogenic to *D. melanogaster*, and one which has been described from these insects[21]; moreover, bacterial community surveys in natural field conditions have demonstrated that Enterobacteriaceae, including the genus *Serratia*, are found naturally in the wild and within naturally occurring populations of *Drosophila*[28]. Therefore, we can hypothesize that the activation of the *Drosophila* immune response by certain strains of bacteria indicates that these bacteria have some natural interaction with the fly, and that these bacteria can perhaps exploit *Drosophila* as a potential intermediate host as well as a vector between fruits, vegetables, or other plants. We also tested other naturally occurring, non-pathogenic bacteria, such as *Acetobacter pomorum* and *Lactobacillus plantarum*, neither of which have been shown to induce substantial immune responses, and are the dominant bacteria strains within the midgut and hindgut of *D. melanogaster* adults and larvae[29].

In previous studies, the ability of *Drosophila* to detect and avoid potentially harmful microorganisms in their environment has been elucidated, such as for pathogenic fungi and bacteria[30–33]. These studies have outlined two olfactory (geosmin, Or56a; phenol, Or46a) and a single gustatory avoidance pathway (lipopolysaccharides, Gr66a) that allow the fly to avoid certain pathogens when presented alone. Conversely, and counter to our initial hypotheses, here we show for the first time that flies become strongly attracted toward conspecifics that have become infected by specific pathogenic bacteria. Moreover, we demonstrate that the increased attraction toward infected flies is due to amplified aggregation pheromone emission by infected flies and their feces, and that this increase is mediated by pathogen-induced alterations to immune, hormonal, and metabolic response cascades following infection.

Results

Behavioral response toward sites of infection. We first tested the behavioral response of *Drosophila* in attraction, feeding, and oviposition toward a natural pathogen, the bacterium *P. entomophila* (Fig. 1a–c and Supplementary Fig. 1A–G). While flies did not respond to the odor of *P. entomophila* in an attraction assay (Fig. 1a, b), we could confirm previous findings from Soldano et al. that flies avoid feeding and ovipositing on food sources containing Gram-negative bacterial pathogens (Supplementary Fig. 1A, C). However, we were also interested in whether *Drosophila* can identify and avoid infected conspecifics as these individuals could be another potential source of infection within the population. Therefore, we repeated the behavioral assays but did not present the pathogen alone, but instead tested infected flies or their feces (Fig. 1c). While both oral and systemic infection generated similar results, for consistency, and to ensure similar levels of infection, all flies were systemically infected along the pleural suture line along the mesothorax with growth media containing bacteria or mock infected with growth media only as a control (Fig. 1j). Contrary to our initial expectation, *Drosophila* strongly preferred the odor of infected flies (or feces of infected flies) over that of healthy flies (or their feces) in the attraction assays (Fig. 1c). We repeated these tests of attraction using an alternative behavioral paradigm, and again we were able to observe that flies were significantly more attracted toward the odors from infected flies when compared to those of healthy controls (Supplementary Fig. 1E). In tests with Orco mutant flies, this preference for infected conspecifics and their feces was lost; thus, we concluded that this attraction was due to olfactory cues (Fig. 1c). We gained similar results when we tested the body

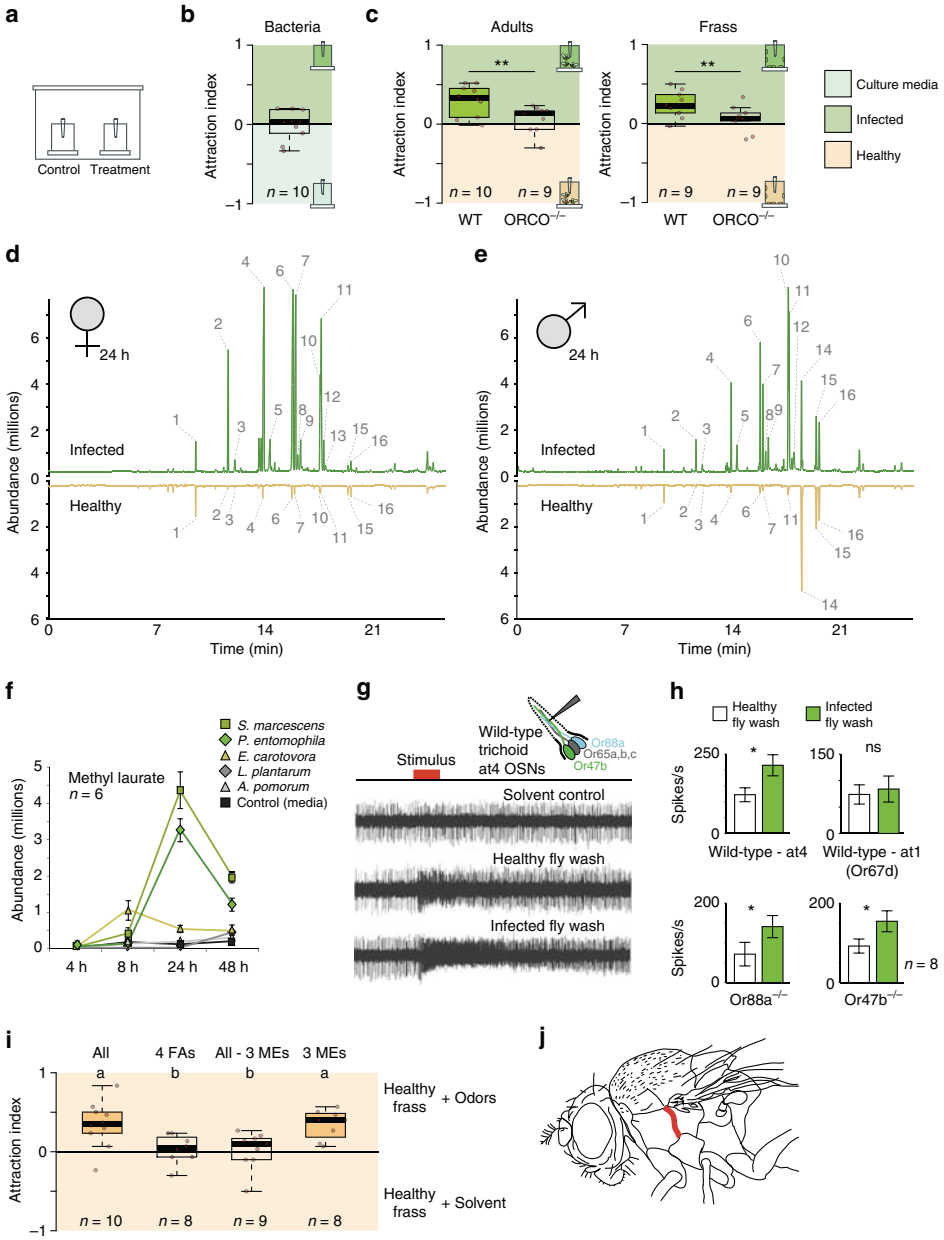

Fig. 1 Effects of infection on *Drosophila* attraction and odor profile. **a** Experimental design of attraction assays. **b** Attraction index of adult *Drosophila* toward the olfactory cues from *Pseudomonas* bacteria or from growth media control. **c** Attraction indices or naive wild type or Orco mutant flies given the choice between other adults with and without *Pseudomonas* infection or between frass of flies with or without infection. Attraction index: ((no. of flies in treatment trap) − (no. of flies in control trap)) / total no. of flies. **d**, **e** GC-MS profile of female **d** and male **e** *Drosophila* adults either infected with *Pseudomonas entomophila* bacteria or mock-infected with growth media (healthy control). Numbers from GC-MS refer to FID peaks: (1) bromodecane (internal standard); (2) methyl laurate; (3) lauric acid; (3) methyl myristate; (5) myristic acid; (6) methyl palmitoleate; (7) methyl palmitate; (8) palmitoleic acid; (9) palmitic acid; (10) methyl linoleate; (11) methyl oleate; (12) methyl stearate; (13) oleic acid; (14) Z-11-cis-vaccenyl acetate (cVA); (15) 7-Z-tricosene; (16) heneicosane. **f** Amount of methyl laurate produced over time, from 4 to 48 h after infection with several strains of bacteria (for time courses of other compounds see Supplementary Fig. 2D). **g** Example of SSR responses of healthy *Drosophila* antennal trichoid (at4) neurons to body washes of infected or healthy *Drosophila*. Stimulus: 0.5 s. **h** Quantified SSR responses toward healthy or infected fly body washes, including recordings from wild-type at4 and at1 neurons, as well as from fly mutants for Or47b and Or88a pheromone OSNs. **i** Attraction indices of adult *Drosophila* toward healthy frass perfumed with treatment odors or solvent control. Frass was perfumed either with all odors (All) that were increased after infection or with a subset. 4FAs: mixture of fatty acids increased after infection that were reported to act as pheromones (lauric acid, myristic acid, palmitoleic acid, and palmitic acid, Lin et al.[36]); 3 MEs: methyl esters (ML, MM, and MP) increased after infection and reported to act as pheromones (Dweck et al.[35]). More details in Supplementary Fig. 2A. **j** Schematic of septic or systemic infection location for both bacterial and mock infection. *Filled boxes* denote significance from zero

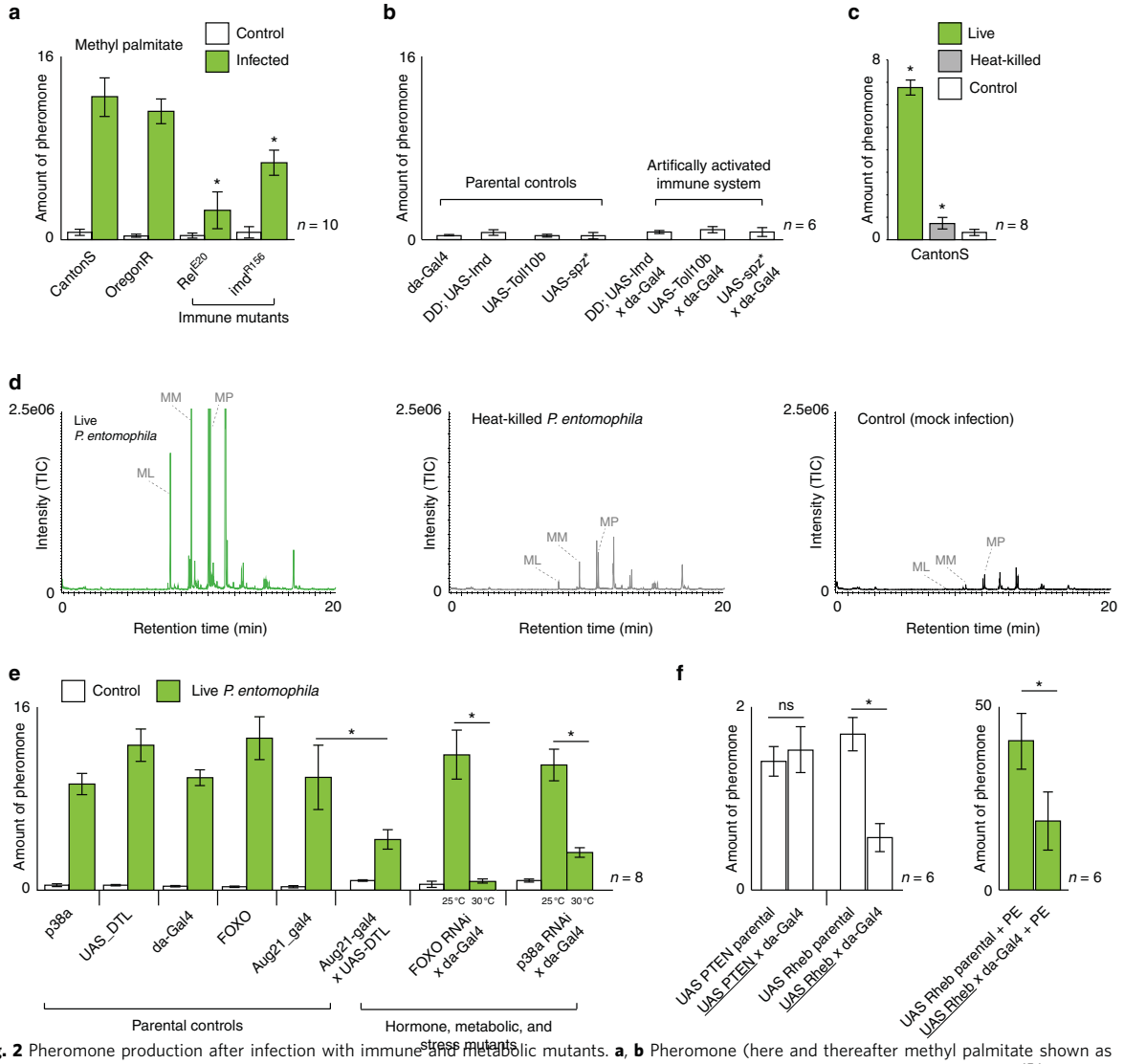

Fig. 2 Pheromone production after infection with immune and metabolic mutants. **a**, **b** Pheromone (here and thereafter methyl palmitate shown as example) production of CantonS or OregonR wild-type flies, and flies deficient in the Imd immune response pathway (RelE20 and IMD^{156}) with *Pseudomonas entomophila* infection (*green*) and without (*white;* **a**), or of flies, where the IMD (Imd flies) or the Toll (Toll flies and spz* flies) response pathway were artificially activated (**b**). **c** Pheromone production after infection with live (*green*) or heat-killed (*gray*) bacteria. **d** GC-MS profiles of live bacteria, heat-killed bacteria, and mock-infected control flies. **e** Pheromone production of flies with either decreased juvenile hormone (Aug21-gal4 x UAS-DTl flies), insulin metabolism (FOXO flies), or stress responses (p38a flies) and their parental control lines. RNAi lines tested at the non-active 25 °C served as further controls. **f** Pheromone production of flies with or without artificial activation of Rheb (an inhibitor of the FOXO pathway) with infection (*green*) or without (*white*)

washes of infected flies or their feces in feeding and oviposition assays (Supplementary Fig. 1b, d). In both cases the flies avoided the bacterium when it was presented alone; however, the flies did not avoid sites of infection and instead preferred infected individuals and material generated by the infected flies (Fig. 1c and Supplementary Fig. 1B, D). Interestingly, the oviposition-related attraction of infected flies was time-sensitive and peaked between 16 and 24 h after infection, while the infected flies were still alive, but dropped after their death (i.e., 48 h after infection, Supplementary Fig. 1D). Thus, it seems that the repulsive behavioral effect of pathogenic bacteria when presented alone can be overcome by the attractive odors generated by infected flies and their feces.

Insect-derived odor emission following infection. In order to examine any odor-derived differences between healthy and infected *Drosophila*, we performed extensive gas chromatography mass spectrometry (GC-MS) analyses of the volatile and non-volatile chemical cues associated with *Drosophila* following systemic infection with pathogenic and non-pathogenic strains of bacteria. While infection with the non-pathogenic *L. plantarum* or *A. pomorum*, or with the facultative endosymbiont *Wolbachia*[34], did not generate any significant difference in the odor profile of the fly (Supplementary Fig. 2C), infection with three strains of natural bacterial pathogens, including *S. marcescens*, *E. carotovora carotovora* (*Pectobacterium carotovora*), and *P. entomophila*, each induced large changes in the chemical profile of

both sexes of infected flies (as compared to mock-infected controls) (Fig. 1d–f; Supplementary Fig. 2C, D). This increase in fly odors after infection included in total 12 compounds (Supplementary Fig. 2A, B). Interestingly, after infection, many of the 12 compounds for which emission increased significantly have been previously identified as *Drosophila* pheromones that modulate courtship and aggregation[35, 36], including methyl laurate (ML), methyl myristate (MM), methyl palmitate (MP), and palmitoleic acid (PA). However, notably, *cis*-vaccenyl acetate (cVA), the male-specific pheromone produced by the male accessory glands, was not affected by any tested bacterial infection (Fig. 1e).

To further examine the increase in pheromone production after infection, we next quantified the amount released over time (Fig. 1f and Supplementary Fig. 2D). After systemic infection with *E. carotovora*, pheromone production peaked around 8 h post infection and returned thereafter to normal levels comparable to those found in control or mock-infected flies. Infection with this strain of bacteria is non-lethal, as the vinegar flies are able to mount a successful immune response to thwart the infection[15]. However, in the case of both *P. entomophila* and *S. marcescens*, pheromone production continued to increase dramatically until the death of the fly, usually around 24 h post infection, with pheromone levels in dead flies then decreasing rapidly toward control levels (Fig. 1f and Supplementary Fig. 2D).

Olfactory response to odors from healthy and infected flies. After having established that pheromone production was highly upregulated in live flies following infection with specific pathogenic bacteria, we proceeded to investigate differences in olfactory responses to this increase in the odor profile of the fly. Using single sensillum recordings (SSRs), we could demonstrate that healthy *D. melanogaster* flies show an increased olfactory response to body washes from infected flies when compared to that of healthy flies (Fig. 1g). We could also show that this response is elicited from olfactory sensory neurons (OSNs) present in the at4 but not in the at1 sensillum (Fig. 1h), and, more specifically, elicited by ligands of the olfactory receptors Or47b and Or88a (i.e., ML, MM, and MP[35]; Fig. 1g, h and Supplementary Fig. 3A–D). Notably, despite PA and several other fatty acids being increased for flies infected with *P. entomophila*, these suggested Or47b ligands[36] did not activate any of the tested OSNs within the at4 sensillum (Supplementary Fig. 3A–D), nor did any of these fatty acids generate a preference in *Drosophila* behavior (Fig. 1i). Together, these results match our previous GC-MS analyses that showed increases after infection for fatty-acid-derived ligands (detected in at4 trichoid sensillae) but not in cVA (detected in at1 sensillum). Moreover, we could show that three fatty-acid methyl esters (ML, MM, and MP) were necessary and sufficient to account for the increased behavioral attraction and electrophysiological response following infection of *Drosophila* with *P. entomophila* bacteria (Fig. 1I and Supplementary Fig. 3A–D).

Pheromone changes with immune and metabolic cascades. Since the pheromone production over time closely matches the published timeline of the immune response to infection for *E. carotovora* and *P. entomophila*[15, 20], we next focused on repeating the GC-MS experiments with immune, hormonal, and metabolic *D. melanogaster* mutants in order to identify any involvement of these pathways in the increased production of pheromones following infection by these bacterial pathogens. Healthy flies with a reduced immune induction (e.g., Rel^{E20} and Imd^{R156} flies)[37] produced normal amounts of pheromones relative to Canton S, but following infection, the same flies produced significantly less pheromones compared to infected wild type (WT) and other control flies (Fig. 2a). This suggests that a functional Imd pathway is necessary for the increase in pheromone production following infection. Moreover, we found that impairment of either the Imd or the Toll immune response pathway resulted in a lower maximum amount of pheromone production after infection with *P. entomophila* (Supplementary Fig. 4A). However, when we tested flies that had either their Imd or Toll immune response pathways artificially activated in the absence of bacteria, we could not induce this increase in pheromones (Fig. 2b), suggesting that the immune system is necessary but not sufficient to account for the change in pheromone production following *P. entomophila* infection. Infection with dead, but intact bacteria can still result in an immune response, including the increase of antimicrobial peptides (AMPs) such as diptericin and drosomycin[15, 19, 38]. We therefore tested whether an infection with heat-killed *P. entomophila* was sufficient to yield AMPs (Supplementary Fig. 4B). Although heat-killed bacteria resulted in the production of two different AMPs and a smaller but significant increase in pheromone production, infection with heat-killed bacteria never reached the degree of pheromone production observed in flies infected with living bacteria (Fig. 2c, d). This suggests that ongoing bacterial growth and subsequent damage by the pathogen are required to induce the large increases in pheromone production.

In addition to the immune response, the fly hormonal system as well as metabolic and stress responses are also affected by bacterial infection, especially in relation to the utilization of the fat body, inflammation, and the mobilization of energy to combat infection, which primarily results in a decrease in adult fat body content[16, 39]. With this in mind, we next focused on the potential origin of these fatty-acid pheromone odors (ML, MM, and MP) by using transgenic fly lines that were deficient in their ability to synthesize juvenile hormone (Aug21-Gal4 > UAS-DTI), flies that were deficient in the transcription factor FOXO (a transcription factor related to insulin signaling and induced in response to stress, pathogenic damage, and starvation), as well as flies deficient in the stress response pathway regulator p38a. Alterations of some of these pathways can be lethal during fly development; thus, in these cases we took advantage of RNA interference (RNAi) inducibility to pass fly development and still test the function of otherwise lethal genes in adult *Drosophila*. The reduction of juvenile hormone through the UAS-Gal4 system, or FOXO via RNAi, caused a significant decrease in pheromone production after infection when compared to the parental lines or to the genetically identical RNAi controls that had not been activated by temperature (Fig. 2e). As p38 directly phosphorylates FOXO[40], these two mutants have already been shown to be linked in their function. Hence, by repeating the experiments with p38a RNAi flies, we were able to confirm the involvement of FOXO in the increased pheromone production after infection. As inhibiting the FOXO transcription factor (either directly through FOXO RNAi or indirectly through p38a RNAi) revealed the most drastic reduction in pheromone production after infection (Fig. 2e), we next activated the *Rheb* gene (part of the target of rapamycin signaling pathway, and which is an inhibitor of the product of FOXO)[17, 41, 42]. As we expected, activating Rheb (and by that indirectly decreasing the product of FOXO), we again found a significant decrease in pheromones, even in the absence of any infection, as well as a strong decrease in infected flies relative to the infected controls (Fig. 2f), thus reconfirming the involvement of FOXO in the pathogen-induced pheromone production. However, when we tested flies in which we increased the expression of PTEN, a factor that is only distantly related to the FOXO transcription factor within the insulin receptor pathway (InR), we did not find any effect on pheromone production (Fig. 2f). Hence, it appears that

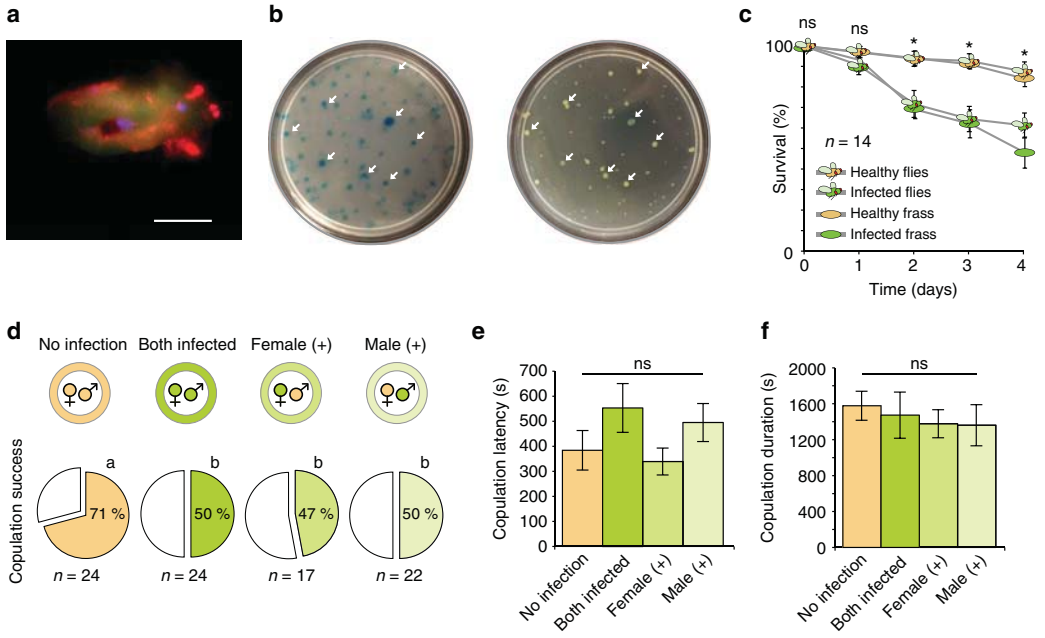

Fig. 3 Ecological impact of preference for infected flies and frass. **a** Frass droplet from a fly that was fed green fluorescent protein (GFP)-labeled bacteria, showing live bacteria (*green*) and dead bacteria (*red*) present in the feces. *Scale bar* depicts 10 µm. **b** Flies fed with a solution containing bacteria and blue dye were allowed to deposit frass onto an agar plate (*left*), and bacterial colony growth was assessed from the fecal deposits (*right*), demonstrating that bacteria can survive the digestive tract and be transferred via feces to new locations. **c** Survival over time of cohorts of flies reared in containers that held either healthy or infected fly adults or their frass (see Supplementary Fig. 5). **d**–**f** Copulation success (**d**), latency (**e**), and duration (**f**) of single pairs of flies following all combinations of infection

several but not all genes related to this metabolic cascade may be influenced by *P. entomophila* infection. When testing oviposition with body washes of flies that were either deficient in their immune response (Relish) or metabolic response (FOXO), we observed a reduced preference for infected flies (Supplementary Fig. 1H). As both immune (Relish) and insulin response pathway mutants (FOXO) resulted in reduced pheromone production after infection, and a corresponding decrease in behavioral preference following infection (compared to WT-infected flies), we conclude that both of these general signaling cascades (immunity and insulin metabolism) are required for *P. entomophila* to alter the fatty-acid pheromone production of *D. melanogaster* adults.

Ecological effects of pheromone changes after infection. We next examined the potential costs and benefits of increased pheromone production for both the insect and the bacteria. Our analyses of fecal material using green fluorescent protein-labeled bacteria revealed that ingested bacteria can survive the digestive tract (Fig. 3a), which was similar to studies that confirmed that yeast can survive ingestion by *Drosophila* and be passed through fecal deposits[43]. In addition, by using blue dye in feeding solutions, we could show that frass deposited on agar plates by infected flies (Fig. 3b, *left*) resulted in new bacterial colonies at the same locations (Fig. 3b, *right*), providing further support that pathogenic bacteria can survive passage through the *Drosophila* digestive system and be transferred to new locations via the oral–fecal route. To study the transmission of bacteria through infected frass material, we introduced healthy flies to containers that held infected conspecifics or to containers in which flies were removed but their frass remained (Supplementary Fig. 5A–D). In both cases we could observe an acute increase in the mortality of the introduced flies when in the presence of infected conspecifics or infected frass (Fig. 3c). Thus, the *P. entomophila* pathogen survives the *Drosophila* gut and potentially profits from increased contact and dispersal through increased attraction of healthy flies toward infected flies or their frass, material that has been previously shown to be attractive for *Drosophila* adults[44]. Moreover, this attraction to infected flies has a high cost for the arriving flies, as they run an increased risk of becoming infected and dying. Conversely, the same attraction could be beneficial for the infected flies, as it could increase their chances of finding a mate and reproducing before death. Thus, we conducted mating assays where all combinations of healthy and infected flies were tested (Fig. 3d). When we presented a healthy and an infected female to two males, preliminary experiments indicated increased orientation and courtship displays toward the infected female; however, in single-pair mating experiments, infection always resulted in lower copulation success, irrespective of whether the female, the male, or both flies were infected (Fig. 3d–f). We furthermore found that infected females were less likely to accept any male for copulation, as they were less likely to extend their abdomen or separate their wings during the male courtship song. We thus found no benefit to the infected fly with regard to successful copulation, even given the robust increase in pheromone production, perhaps due to other confounding behavioral alterations after infection, such as lethargy, cell damage, or another byproduct of pathogen growth. Although the increased pheromone emission did not result in the hypothesized higher mating success of infected flies, we cannot exclude that without this increase infected flies would even have less copulation. It is also possible that different degrees of infection may result in increased courtship success, although additional work is

required to address this hypothesis. Therefore, our current data suggest that the increased pheromone emission of infected flies mainly results in attracting more *Drosophila* to sites of infection, promoting contact and dispersal benefits for the bacterial pathogens, while not providing any direct courtship benefit to the infected fly.

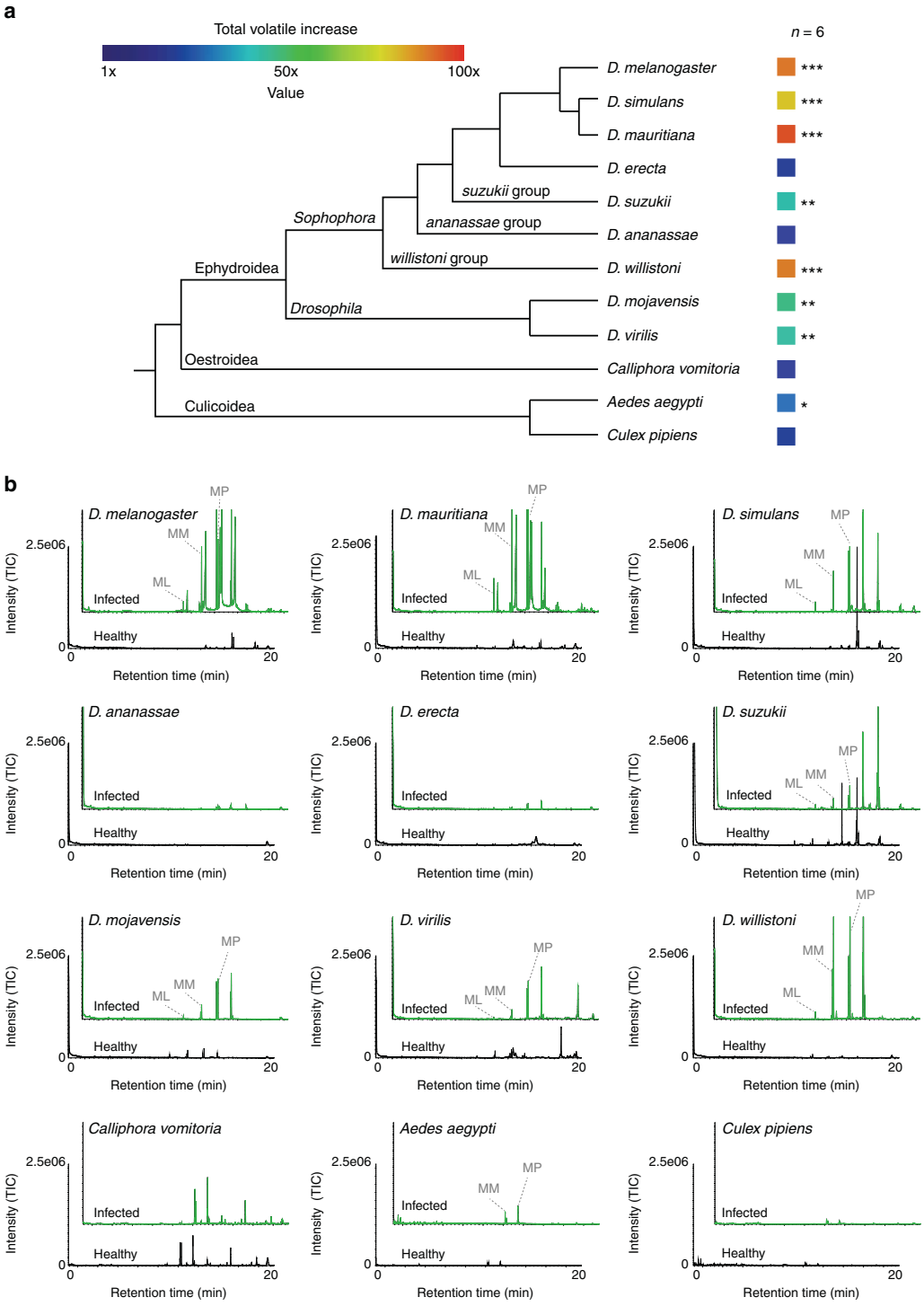

Fig. 4 Infection of *Drosophila* and Diptera species with *P. entomophila* bacteria. **a** Phylogenetical relationship and color-coded relative increase of odor emissions after infection for all tested species. **b** Example of GC-MS traces for each species before and after infection with *P. entomophila* bacteria with those methyl esters identified that were behaviorally relevant in *D. melanogaster*

Pathogenic infection with other Dipterans. To augment our screening of *D. melanogaster*, we also tested *P. entomophila* infection with eight other Drosophilids and three other Dipterans, including the blue bottle fly, *Calliphora vomitoria*, as well as two mosquitoes, *Aedes aegypti* and *Culex pipiens* (Fig. 4a, b). While infections were lethal for all tested insect species, we found significantly increased emissions of potential fatty-acid pheromones in seven out of nine *Drosophila* species as well as in *A. aegypti* (but no increase in *Calliphora* nor in *Culex*), suggesting that the manipulation of the insect's volatile emission by the pathogen *P. entomophila* is a more general phenomenon.

Discussion

We conclude that specific pathogenic bacteria can overcome the avoidance mechanisms of *D. melanogaster* flies[32] by taking advantage of, or hijacking, a chemosensory circuit related to social communication[35, 36, 45]. This preference and attraction toward infected individuals is due to pheromone signals and cannot be avoided by conspecific flies, as these chemical cues are vital for both aggregation and courtship in *D. melanogaster*. While previous research has documented viral or parasitic alterations in pheromone production for *Helicoverpa zea* and *Apis mellifera*[7, 46], the ecological impact as well as physiological and neural mechanisms for this shift have not been previously addressed. Here we assert that both the immune response pathway and the InR pathway are necessary for this increase in fatty-acid-derived pheromone release after infection by *P. entomophila* bacteria. This linkage between the *Drosophila* immune system, insulin signaling, and the fat body has been previously noted[39], as has the connection between the Rheb, FOXO, and damage response pathways[41]. However, our data show for the first time a pheromone change in *Drosophila* after infection, and show a mechanistic connection between the pathogen and the alteration of the pheromone communication system of the insect host. In addition, our data also reveal for the first time the associated ecological ramifications for both the pathogen and for the insect following infection.

This increase in pheromone production after infection might just be a byproduct of the bacterial growth and the associated damage to the insect[16, 39]; however, this insect–microbe interaction results in a potential evolutionary advantage for the bacterium by increasing its chances for contact and dispersal through enhancing several aggregation pheromones of a potential host and insect vector. Previously, it has been suggested that humans infected with malaria are more attractive to the *Anopheles* vector and that mosquito vectors carrying Malaria are also more likely to take additional bloodmeals, both of which result in increased dispersal benefits for the *Plasmodium* protozoan[47–50]. Our data may be pertinent for not only the study of insect-transmitted human diseases, but also studies related to insect-vectored plant pathogens, such as those similar to the *Drosophila*-transmitted plant pathogen *E. carotovora* used in this study. In addition, the application of species-specific pathogens may be useful as a tool in identifying novel pheromones from other infected host organisms, such as *D. suzukii* or *A. aegypti*.

Therefore, in summary, it is our assertion that specific pathogenic bacteria alter the lipid metabolism of *Drosophila* during infection through both immune and insulin signaling pathways, which results in increased fatty-acid pheromone release by the adult insect after infection. Moreover, this increase in pheromone release attracts more adult flies to sites of infection and contributes to the potential uptake and dispersal of the pathogenic bacteria toward new fruit, vegetable, or insect hosts. Thus, our data begin to generate a better understanding of how microorganisms can alter insect host physiology as well as alter insect host behavior, and, moreover, our findings might provide future tools or novel strategies to combat insect-transmitted human and plant diseases.

Methods

Drosophila stocks. WT fly lines included the *D. melanogaster* Canton-S and OregonR strains. Flies were raised on standard diet at 25 °C with a 12 h light/dark cycle. Transgenic lines related to immunity, hormones, and insulin signaling were obtained where possible from the Bloomington Drosophila Stock Center (flystocks.bio.indiana.edu), and include: *p38a RNAi*, *Rel*^{E20}, *DD; UAS-imd, UAS-Toll10b*, *FOXO RNAi*, *IMD*^{R156}, *UAS-Rheb* (BL 9690), *Aug21-Gal4, UAS-DTI, UAS-spz**, and *da-Gal4* (Gaia). Other transgenic lines include: *Or88a* mutant (Leslie Vosshall; E4365-181) and *Or47b*[3] mutant (BL 51307). All fly lines have been described previously[15, 19, 35, 37, 41]. *Drosophila* RNAi lines after crossing were kept at 30 degree (treatment) or 25 degree (as negative controls) prior to subsequent testing with infection.

Bacterial strains and infection experiments. Bacterial strains were kept in long-term storage at −80 °C in 70% glycerol or 70% dimethyl sulfoxide (DMSO). Fresh bacterial cultures were generated daily and cultured overnight in 1000 µl lysogeny broth (LB) growth medium and grown at 29 °C and 70% humidity[51]. Adult flies between 4 and 7 days of age were pricked with a sharpened tungsten needle that had first been sterilized with ethanol and then inoculated by dipping the needle into a concentrated bacterial pellet[52]. Control flies were also pricked in the same manner, but with only LB culture medium. Flies were maintained for set time intervals at 29 °C following infection with either the bacteria or the mock control and then later used for subsequent behavioral experiments or body wash collections. To generate heat-killed samples, fresh 1 ml bacterial cultures were placed into Eppendorf tubes and then allowed to float in a water bath that was heated to 90 °C for 1 h. After cooling to room temperature, these heat-killed bacteria were then used following the previously described pricking procedures to infect the adult flies. Bacteria were also confirmed to be dead by plating them without observing any growth.

Trap assays and FlyWalk. Trap assays were performed with 2–5-day-old flies as previously described[44, 53]. Briefly, test chambers (transparent yoghurt cups (500 ml) with 50 ventilation holes in the lid) contained a treatment and a control trap made from small transparent plastic vials (30 ml) with a cut micropipette tip (tip diameter 2 mm) inserted into a hole of the vial. Thirty flies (males and females, ratio about 1:1, 4–5 days old, starved for 24 h before the experiment) were placed in each test box. Experiments were always started at the same time of day and carried out in a climate chamber (25 °C, 70% humidity, 12-h-light:12-h-dark cycle). The number of flies in and outside the traps was counted after 24 h. Valence of the tested cuess was quantified with an attraction index (AI), calculated as: $AI = (O - C)/(30)$, where O is the number of flies in the odorant trap, C the number of flies in the control trap, and 30 the sum of all flies tested. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). A value of zero characterizes a neutral or non-detected odorant. FlyWalk trials were also conducted as described previously[54, 55]. In short, 15 individual flies were placed in glass tubes (0.8 cm i.d.). The glass tubes were aligned in parallel, and flies were monitored continuously by an overhead camera. *xy* positions were recorded automatically at 20 fps using Flywalk Reloaded v1.0 software (Electricidade Em Pó; flywalk.eempo.net). Experiments were performed under red LED light (peak intensity at λ, 630 nm). During the experiments, flies were continuously exposed to a humidified airflow of 20 cm/s (70% relative humidity, 20 °C). Flies were repeatedly presented with pulses of various olfactory stimuli at interstimulus intervals of 90 s. Stimuli (i.e., headspace of either 100 healthy or infected adult flies (50 males and 50 females)) were added to the continuous airstream and thus traveled through the glass tubes at a constant speed. The paradigm allows us to measure the stimulus-induced change of upwind speed of the tested flies.

Feeding assays. Flies were collected and tested between the ages of 2–5 days, and included both males and females that were starved beforehand for 18–20 h with constant access to water. Flies were then cooled for 2 min at −20 °C to assist in their transfer to the behavioral arena. The capillary feeder (CAFÉ) assays utilized glass micropipettes with liquid media that were filled by capillary action and then inserted through pipette tips into the container holding the adult flies, modified from Ja et al.[56]. One capillary contained the control (5% sucrose with LB media), while the other contained the treatment (5% sucrose plus LB media and either bacteria or frass), and the volume consumed from each side was measured after a set duration of feeding. Feeding indices were calculated as $(T - C)/(T + C)$, where T is the amount of food consumed from the treatment solution and C is the amount of food consumed from the control solution.

Chemical analyses and SSRs. All of the synthetic odorants that were tested and confirmed were acquired from commercial sources (Sigma, www.sigmaaldrich.com, and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery for behavioral experiments followed

previously established procedures, and collection of volatile and non-volatile compounds was carried out according to standard procedures[35, 44]. GC-MS (HP5 and HP-Innowax) and TDU-GC-MS analyses were performed on all odor collections and insect body washes as described previously[35]. The NIST mass-spectral library identifications were confirmed with chemical standards where available, and the internal standard bromodecane was utilized for quantification and statistical comparisons between analyzed samples. SSR experiments were conducted as described previously[35, 44].

Oviposition experiments. Virgin flies were collected and separated by sex 4–5 days prior to the experiments. Before the trials, male and female virgins were allowed to mate for 4 h, and then separated again. Cohorts of 20 recently mated females were added to small container (10 × 10 × 20 cm) that was equipped with two Petri dishes (diameter, 5 cm) containing agar (1%), of which one was loaded with the odor in solvent, and the other with solvent only (or with another odor, if, e.g., when odors of infected vs. healthy flies were tested). Experiments were carried out in a climate chamber (25 °C, 70% humidity, 12 h light:12 h dark cycle). We added 50 µl of body wash extracts collected from either healthy (mock infection with LB media) or body washes from flies infected with *P. entomophila* for sequential time intervals. Flies were allowed to lay eggs for 3 days, after which eggs were counted to generate the oviposition indices (which were calculated as $(T - C)/(T + C)$ where T is the number of eggs on the treatment plate and C is the number of eggs on the control plate).

Courtship and mating experiments for single pairs. Adults were collected as newly emerged virgins, where males were kept in individually separated vials and females were reared in groups of 20–30 flies. Courtship was conducted with virgin flies that were 4–5 days old, and the behavioral experiments were conducted as described previously within the lid of an Eppendorf that was covered by a plastic slide[35]. Mating and courtship behaviors were recorded for 20 min and then analyzed. Copulation latency refers to the time delay until the successful physical coupling of the male and female, while copulation success refers to the percentage of total pairs that mated within the 60 min timespan. Copulation duration was the time that the male and female were conjoined during mating.

Statistics and figure preparation. Statistical analyses were conducted using GraphPad InStat 3 (https://www.graphpad.com/scientific-software/instat/), while figures were organized and prepared using R Studio, Microsoft Excel, and Adobe Illustrator CS5. The Wilks–Shapiro test was used to determine normality of each data set. Normally distributed data were then analyzed using two-tailed, paired *t*-tests and one-way analyses of variance. Nonparametric distributed data were assessed using Kruskal–Wallis with Dunn's post hoc test for multiple comparisons for selected pairs. An asterisk denotes statistical significance between two groups ($^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Courtship data were analyzed and confirmed by an additional blind observer who was not aware of the treatments being viewed. Boxplots represent the median (bold black line), quartiles (boxes), as well as the confidence intervals (whiskers). Whiskers in barplots represent the standard error.

Data availability. Additional supplementary information and extended data including methodology, courtship videos, and other raw data are available with the online version of the publication. All data supporting the findings of this study are available within the article and its Supplementary Information files.

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Author contributions

I.W.K., M.K., N.B., and B.S.H. contributed to the design of this study. I.W.K. conducted the behavioral trials including trap and CAFÉ feeding assays. I.W.K. and S.K. performed the oviposition and heat-killed bacteria assays, while S.K. and N.B. designed the *Drosophila* mutant and RNAi experiments. M.A.K. and I.W.K. completed the courtship and mating assays, as well as the SSR data sets. T.R. conducted the flywalk trials. I.W.K. conducted all the GC-MS experiments, while M.A.K. and I.W.K. completed the TDU-GC-MS experiments. I.W.K. prepared the original manuscript, and M.K., N.B., and B.S.H. contributed to the final manuscript and subsequent revision.

Additional information

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Manuscript IV

Odor mixtures of opposing valence unveil interglomerular crosstalk in *Drosophila* antennal lobe

Ahmed A. M. Mohamed, Tom Retzke, Sudeshna Das, Bill S. Hansson, Markus Knaden and Silke Sachse

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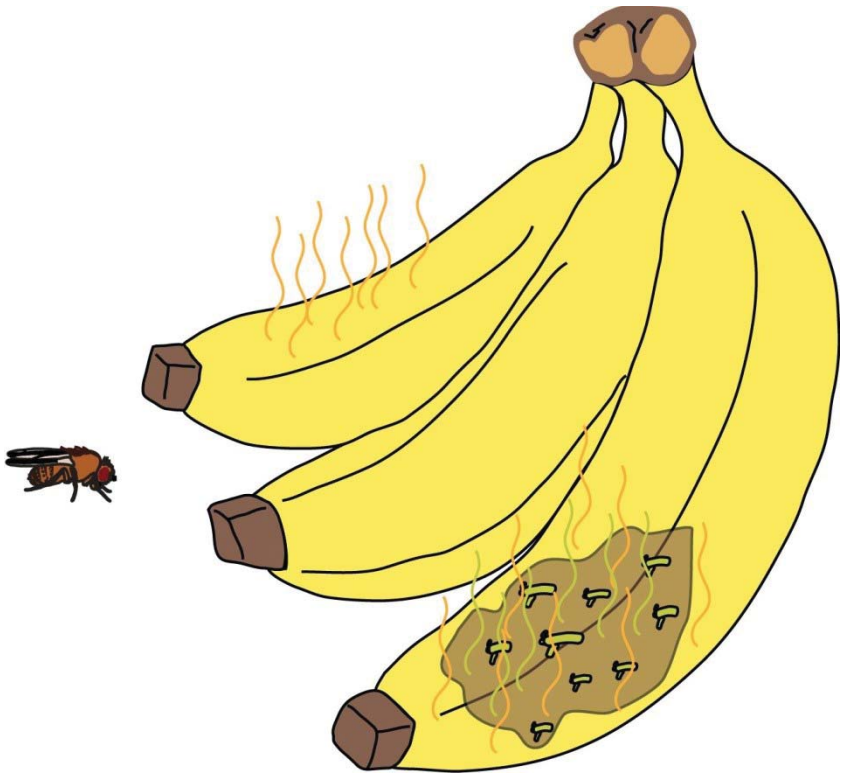

Odor mixtures of opposing valence unveil inter-glomerular crosstalk in the *Drosophila* antennal lobe

Ahmed A. M. Mohamed[1], Tom Retzke[1], Sudeshna Das[1], Bill S. Hansson[1], Markus Knaden[1,*] and Silke Sachse[1,*,#]

[1]Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, 07745 Jena, Germany

[*]Share senior authorship

[#]Corresponding Author: Silke Sachse, Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, 07745 Jena, Germany; phone: +49-3641-1405, fax: +49-3641-1401, email: ssachse@ice.mpg.de

Abstract

Insects use their sense of olfaction to locate, among others, potential food sources like fruits. In general those do not emit only single compounds, but a mixture of odors. These blends can occur in several constellations regarding the valence of the compounds. With this study we show behavioral responses of *Drosophila melanogaster* to binary mixtures consisting of odors of opposing valence, how these mixtures are processed in the antennal lobe and which networks are involved. In behavioral experiments we observed attraction to or repulsion from the mixture depending on the ratio between the single components. We found the same ratio-dependency in the antennal lobe when we did calcium imaging of odor-specific glomeruli. Thus we show inhibition of individual glomeruli on the projection neuron level. Further we show by drug application that this inhibition is mediated by GABA. We could confirm that this neurotransmitter is released by a subset of GABAergic LNs within the antennal lobe

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Introduction

An important role of an animal's brain is to encode, integrate and interpret the vast array of olfactory stimuli in the surrounding environment in order to turn this sensory input into a behavioral output. However, most, if not all, odors encountered by the animal are not single molecular compounds but, rather, complex blends varying in the valence and ratios between their individual components. Hence, the olfactory system is confronted with the goal of evaluating the whole mixture, not individual components, to obtain the ecological relevance for the animal.

The simplicity and the stereotypic organization of the olfactory system of *Drosophila melanogaster* make it a favorable model to study, behaviorally and physiologically, odor mixture interactions. An adult *Drosophila* detects odors with olfactory receptor neurons (ORNs) housed in olfactory sensilla on the antennae and the maxillary palps (Stocker, 1994; Vosshall and Stocker, 2007). Most of the ~50 ORN types express one (or two) odorant receptors (ORs) together with the co-receptor (Or83b, Orco) (Benton et al.,

2009; Couto et al., 2005; Fishilevich and Vosshall, 2005; Larsson et al., 2004; Silbering et al., 2011; Vosshall and Hansson, 2011). All of the ORNs expressing the same OR innervate the same glomerulus in the antennal lobes (ALs) (Couto et al., 2005; Vosshall, 2000), where they synapse onto second-order neurons (projection neurons, PNs) (Stocker et al., 1990). Glomeruli are interconnected to each other by local interneurons (LNs) which are mostly GABAergic and synapse onto both ORNs and PNs (Ng et al., 2002; Silbering et al., 2008; Tobin et al., 2017; Wilson and Laurent, 2005; Wilson et al., 2004).

The global anatomy of most LNs supports the idea of global lateral inhibition (Chou et al., 2010; Hong and Wilson, 2015; Ng et al., 2002; Sachse and Galizia, 2002; Seki et al., 2010). Nonetheless, some of the inhibitory LNs are shown to connect specific glomeruli and are believed to mediate specific crosstalk between those glomeruli (Chou et al., 2010; Okada et al., 2009; Seki et al., 2010). Most of the lateral inhibition in the AL occurs on the presynaptic locus and is mediated via $GABA_A$ and $GABA_B$ receptor types (Olsen and Wilson, 2008; Root et al., 2008). A weaker lateral inhibition takes place at the PN level (Ng et al., 2002; Shang et al., 2007; Wilson and Laurent, 2005; Wilson et al., 2004).

Composition and concentration of each component within an odor mixture has been reported to influence mixture interactions (Shen et al., 2013). Previous studies have shown that mixture interactions can take place at the ORN level (Deisig et al., 2012; Hillier and Vickers, 2011; Münch et al., 2013; Pregitzer et al., 2012; Schuckel et al., 2009; Su et al., 2011; Su et al., 2012). AL responses to mixture stimulations are qualitatively predictable from the responses to the single compounds of the mixture (Carlson, 1996; Deisig et al., 2006; Deisig et al., 2010; Olsen et al., 2010; Silbering and Galizia, 2007). A recent behavioral study even showed that one can predict the behavioral output to a mixture based on the responses towards the mixture constituents (Thoma et al., 2014). Another behavioral study showed that artificial co-activation of two single specific ORNs, which individually evoked attraction behavior, results in either the sum of the component responses (so-called summation) or is resembles the response to the larger component (s-called max pooling) (Bell and Wilson, 2016). When an

aversive-coding ORN (Gr21a) was co-activated with one of the attractive ORNs, the attraction was significantly reduced (Bell and Wilson, 2016). However, how does the neuronal circuitry of the olfactory system accomplish such mixture processing and how does that correlate with the behavioral output?

In order to analyze the neuronal processes of mixture interactions in the fly AL, we addressed the following four questions: First, how does a fly behave in response to a mixture of attractive and aversive odors? A recent study showed that mice receive a mixture of attractive and aversive odors as neutral or, in some cases; the mixture turns to complete aversion (Saraiva et al., 2016). Second, how does the *Drosophila's* olfactory system integrate the information of a mixture of attractive and aversive odors? Mixture interactions in the olfactory systems of vertebrates and invertebrates have been well reported (Deisig et al., 2006; Gupta et al., 2015; Olsen et al., 2010; Shen et al., 2013; Silbering and Galizia, 2007; Su et al., 2011; Su et al., 2012; Tabor et al., 2004). However, few of these studies focused on the hedonic valence of the mixture constituents. Third, what is the neuronal mechanism behind the mixture interaction in the AL? Most of the mixture interactions are caused by lateral inhibition of some glomeruli mediated by GABA receptors (Olsen et al., 2010; Silbering and Galizia, 2007) which can take place at the presynaptic and/or postsynaptic loci (Olsen and Wilson, 2008; Root et al., 2008; Silbering and Galizia, 2007; Silbering et al., 2008; Wilson and Laurent, 2005). Finally, fourth, is there any specific inhibitory cross-talk between specific glomeruli? Lateral inhibition was shown to be global in the AL (Asahina et al., 2009; Hong and Wilson, 2015; Olsen et al., 2010), specific or sparse (Girardin et al., 2013; Ng et al., 2002), or both (Sachse and Galizia, 2002; Silbering and Galizia, 2007; Silbering et al., 2008). LNs vary in their innervation patterns: Most of them innervate all or most of the glomeruli, others innervate few glomeruli which are thought to mediate specific interactions between those glomeruli (Chou et al., 2010; Okada et al., 2009; Seki et al., 2010).

In this study, we took behavioral and physiological approaches to address the mechanistic question of how the fly reacts to a binary mixture of attractive and aversive odors. We found that the behavior and physiology of mixture responses vary with

different identity and concentration ratios of the mixture constituents. We also demonstrated that lateral inhibition caused by mixture interactions affects only those circuits that encode attractive odors with no obvious inhibition on the aversive channels. Interestingly, certain glomeruli that are activated by attractive odors (Knaden et al., 2012) showed differences in the level where the inhibition to the binary mixture takes place. Some glomeruli are predominantly inhibited on the presynaptic locus, others are inhibited, interestingly, on both the pre- and the postsynaptic level. Moreover, we found that some of the aversive glomeruli have specific interglomerular interactions with specific attractive glomeruli. We conclude that mixture interactions of opposite hedonic valence takes place in an early olfactory processing center (i.e. the ALs), and is mediated by specific interglomerular cross-talk.

Results

Determining the behavioral responses to binary mixtures of opposing valences

We first addressed the question of how flies behave in response to binary mixtures of opposing hedonic valences. To answer this, we used the FlyWalk (Steck et al., 2012), a behavioral bioassay that monitors odor-guided walking behavior. In this assay, individual freely walking flies were placed in glass tubes, mimicking small wind tunnels, and each individual fly was tracked automatically while presented with different olfactory stimuli (Figure 1A). As a starting point for this study, we picked ethyl acetate (ETA) as an attractive odor for adult flies (Beshel and Zhong, 2013; Keller and Vosshall, 2007; Larsson et al., 2004; Thoma et al., 2014) and larvae (Khurana and Siddiqi, 2013; Kreher et al., 2008) and benzaldehyde (BEA) as an aversive odor (Knaden et al., 2012; Steck et al., 2012; Wasserman et al., 2012). We used a dilution of (10^{-1}) of benzaldehyde because it was shown to be highly aversive to flies, (10^{-2}) dilution of ethyl acetate and the binary mixture of both. In this experiment, flies showed the same attraction to the mixture (hereafter MIX (+)) than to ETA alone (Figure 1B-1D). While keeping the concentration of the aversive odor the same (benzaldehyde, 10^{-1}), we used a more diluted ethyl acetate (10^{-3}) and their binary mixture. We found the attraction towards the

mixture (hereafter MIX (-)) was significantly reduced compared to ethyl acetate alone (Figure 1E-1G). These results imply that *Drosophila* adults behave differently towards mixtures with different ratios of attractive and aversive odors, and that the repellent odor has an impact on the fly's behavior dependent on the concentration of the attractive odor.

MIX (-) induces inhibition in individual glomeruli at the PN level

Ethyl acetate and benzaldehyde drive activity in, mostly, non-overlapping ORN types (de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2008; Schlief and Wilson, 2007) (Figure S2A and S2B). We next monitored the output signal of the AL upon stimulation with the binary mixtures and their constituents to analyze whether any mixture processing in form of lateral excitation (Olsen et al., 2007; Shang et al., 2007) and/or lateral inhibition (Olsen and Wilson, 2008; Root et al., 2008; Wilson and Laurent, 2005; Wilson et al., 2004) is taking place in the AL. We expressed the genetically encoded calcium sensor GCaMP6s (Chen et al., 2013) in PNs under the control of *GH146-Gal4*, which labels most of the uniglomeruli PNs (Grabe et al., 2015; Stocker et al., 1997). We then used two-photon microscopy (Wang et al., 2003) to image odor evoked signals in PNs. We have established an odor delivery system, similar to the one used in the Flywalk, attached to the two-photon microscopy (Figure 2A). We verified the efficiency of the odor delivery system using a photoionization detector (PID) and SPME GC-MS (Figure S1A and S1B). Hence, the identical odors with the same concentrations used in the Flywalk experiments were applied in the functional imaging experiments. We identified individual glomeruli based on their anatomical position using the in vivo 3D AL atlas (Grabe et al., 2015) (Figure S2). Using the high concentration of ethyl acetate (10^{-2}), benzaldehyde (10^{-1}) and their mixture MIX (+), we did not observe any differences neither in the response patterns nor in signal intensities between the responding glomeruli to the mixture and its corresponding individual odorants: i.e. all activated glomeruli showed the same response intensities to stimulation of MIX (+) or the individual components, while no additional glomeruli were recruited (Figure 2B; 2D and S2A).

Next we measured the calcium responses to the lower concentration of the ethyl acetate (10^{-3}), the same concentration of benzaldehyde as in the previous experiment (10^{-1}), and their binary mixture (MIX (-)), which showed a low attraction behaviorally (Figure 1). Interestingly while there was no difference in the activity patterns to the mixture (Figure S2B), we noticed a strong inhibition in those glomeruli that responded to ethyl acetate (henceforward *attractive* glomeruli DM1, DM2, DM3 and DM4) towards the MIX (-) compared to their activity to the attractant alone. However, glomeruli that were activated to the repellent odor benzaldehyde (henceforward *aversive* glomeruli DL1 and DL5), revealed the same response intensity to the mixture as compared to BEA alone (Figure 2C; 2E and S2B).

In order to compare the odor representations between the different mixtures, we plotted the odor-evoked calcium signals of the main *attractive* and *aversive* glomeruli (DM1, DM2, DM3, DM4, DL1 and DL5) in a principle component analysis. While the higher concentration mixture (MIX (+)) lies perfectly in between the corresponding attractant and repellent, the lower concentration mixture (MIX (-)) shifted in favor of the repellent odor (no significant difference between benzaldehyde (10^{-1}) and MIX (-), ANOSIM Rho similarity index, Bonferroni-corrected p-values) (Figure 2C). These data indicate that the concentration of the attractant and the repellent in a binary mixture represents the key factor influencing the signal output from the olfactory system.

The ratio between the attractive and aversive odors determines the fly‘s behavior and correlates with the glomerulus-specific inhibition

We next asked whether the behavioral difference between the two different mixture concentrations and the physiological inhibition of the attractive glomeruli for the lower concentration mixture were caused by the ratio between the attractant and the repellent or the net concentration of both odors. First, we monitored the behavioral response in the Flywalk to a lower concentration of the aversive odor (benzaldehyde 10^{-2}), the attractive odor at a low concentration (ethyl acetate 10^{-3}) and the mixture of both. Surprisingly, flies were attracted to this mixture equally strong as to ethyl acetate (10^{-3})

alone (Figure 3A). Hence, the behavioral response corresponds to MIX (+). We then measured the calcium signals from PNs in the AL using the same odor set. Notably, we found that benzaldehyde at the low concentration (10^{-2}) did not cause any inhibition on the *attractive* glomeruli activated by ethyl acetate (10^{-3}) in the mixture (Figure 3B; S2C and S2E). However, by lowering the concentration of the attractive odor to 10^{-4}, which is still highly attractive on its own (Figure 3C), in a mixture with BEA of (10^{-2}), we observed a reduction in the flies' behavioral attraction to this mixture, which we can define as MIX (-). In addition, we observed a strong inhibition in the activity of the *attractive* glomeruli when stimulated with MIX (-) compared to their activity to the corresponding individual odors. (Figure 3D; S2D and S2F). As expected, the *aversive* glomeruli did not reveal any mixture interactions (Fig. 3B, D). Taken together, these results indicate that the ratio between the attractant and the repellent, not the net concentration of both, in a binary mixture is determining the attractiveness of the mixture and the impact of the repellent on inhibiting the attractant's pathway early in the olfactory system, i.e. at the level of the ALs.

Pattern of inhibited glomeruli depends on activated ORN-types

Do aversive circuits generally inhibit the *attractive* glomeruli or do other odor mixtures affect other glomeruli? To answer this question we tested, behaviorally and physiologically, other odor combinations of attractive and aversive odors and their binary mixtures. Our initial aversive odor benzaldehyde activates mainly Or10a and Or7a-expressing ORNs, which target the glomeruli DL1 and DL5, respectively (Couto et al., 2005) (Figure S2A and S2B). We next asked what happens if we only stimulate one of these two benzaldehyde-responsive glomeruli. To do so we applied the odor methyl salicylate (MSC 10^{-3}), which was shown to repel flies in the Flywalk (Figure 4A and 4B) (Thoma et al., 2014) and which solely activates Or10a (i.e. glomerulus DL1) at a certain concentration (Figure 4A'', 4B'', S2C and S2D) (de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004). To evaluate the impact of activating glomerulus DL1 only, which was predicted to mediate negative odor valence (Knaden et al., 2012), we combined methyl salicylate with ethyl acetate at two different concentrations, ETA 10^{-2} and ETA 10^{-3}. While the first mixture did not cause any change in the innate

attraction towards ethyl acetate (corresponding to MIX (+)) (Figure 4A), and also the glomerular responses to the binary mixture quantitatively corresponded to the responses to the single odors (Figure 4A', 4A'' and S2E), though, in the second mixture (MIX (-)), MSC had a strong impact on the attraction of the ethyl acetate at 10^{-3} concentration (Figure 4B). Surprisingly, two (DM1 and DM4) out of the four *attractive* glomeruli (DM1, DM2, DM3 and DM4) were strongly inhibited in response to this binary mixture (MIX (-)) compared to the ethyl acetate responses. The other two glomeruli (DM2 and DM3) were equally activated by the mixture and ethyl acetate alone (Figure 4B' and S2F), while in the case of DM3, this glomerulus was slightly more activated in the mixture. Notably, DL1 was equally activated by benzaldehyde (10^{-1}) and methyl salicylate (10^{-3}) (Figure 2D, 2E, 4A'' and 4B''), which indicates that the differences in the inhibition patterns of the different binary mixtures is due to a different activation of the ORN types activated by the two aversive odors.

Next, we asked whether there are other glomeruli which are activated by the attractive odor and which would be inhibited by a binary mixture of opposite valences. We chose balsamic vinegar (BAL) as it is one of the most attractive odor for vinegar flies (Figure 4C) and activates 3 additional glomeruli (D, DC1 and DC2) (Figure 4C''') beside the four highly activated glomeruli overlapping with ethyl acetate (DM1, DM2, DM3 and DM4) (Figure 4C' and S3G). Behaviorally in the Flywalk, we tested different concentrations of balsamic vinegar mixed with the repellent odor benzaldehyde (10^{-1}) (data not shown) and picked the concentration of balsamic vinegar (10^{-2}) in which its binary mixture with benzaldehyde (10^{-1}) shows a suppression of the attraction (i.e. MIX (-), Figure 4C). We then performed two-photon calcium imaging from PNs using these two odorants and their binary mixture. When DL1 and DL5 were activated by MIX (-) (Figure 4C''' and S3G), we observed an inhibition in the four *attractive* glomeruli as in the ethyl acetate case (DM1, DM2, DM3 and DM4) (Figure 4C'). However, the additional three *attractive* glomeruli (D, DC1 and DC2) were equally stimulated by MIX (-) and balsamic vinegar (Figure 4C'').

Up to this point, we tested aversive odors, which highly activate glomeruli DL1 and/or DL5. Hence, we wondered whether another aversive odor that does not activate these

glomeruli would induce the same inhibitory effect. We chose geosmin (GEO) since it only activates Or56a (targeting glomerulus DA2) and is highly repellent to flies (Becher et al., 2010; Stensmyr et al., 2012). As the attractive odor we selected balsamic vinegar because it activates a higher number of glomeruli. Geosmin at a concentration of (10^{-3}), balsamic vinegar at (10^{-2}) and their binary mixture were used in this experiment (Figure 4D). In consistent to a previous study, geosmin significantly reduces the innate attraction behavior to balsamic vinegar in this mixture (Stensmyr et al., 2012) (Figure 4D). However, PNs of the *aversive* and *attractive* glomeruli showed same activity to this binary mixture and its individual odorants (Figure 4D'- 4D''' and S3H) The lack of this mixture inhibition effect on any of the *attractive* glomeruli can be explained as DA2 (geosmin glomerulus) is one of the few glomeruli that is particularly narrowly tuned beside DA1, VA1d and VA1v, which respond selectively to fly pheromones, and V glomerulus, which responds selectively to carbon dioxide (Clyne et al., 1997; Dweck et al., 2015; Stensmyr et al., 2012; Suh et al., 2004; van Naters and Carlson, 2007). It is a conceivable that narrowly-tuned glomeruli have a different impact on the behavioral output than broadly tuned glomeruli, such as DL1 and DL5.

In sum, these findings demonstrate that activating different ORN types by aversive and attractive odors in a binary mixture induce different inhibitions at the PN level in the ALs. Notably, we did not observe any mixture inhibitions in the *aversive* glomeruli by any of our binary mixture combinations even though at high concentration of the attractive odor. It seems that the aversive neuronal circuity can affect and inhibit the attractive circuity, but not *vice versa*.

Inhibition induced by mixtures of opposing valences is mediated by GABA only and occurs at the PN and ORN level

We next turned our attention to the neuronal mechanism underlying the inhibition of the *attractive* glomeruli caused by the binary mixture of an attractive and aversive odor. Most, if not all, of the odor-induced inhibitions in the *Drosophila* AL is mediated by the inhibitory neurotransmitter GABA which binds to either the fast ionotropic $GABA_A$

receptors, the slow metabotropic $GABA_B$ receptor or to both (Christensen et al., 1998; Harrison et al., 1996; MacLeod and Laurent, 1996; Olsen and Wilson, 2008; Raccuglia et al., 2016; Root et al., 2008; Sachse and Galizia, 2002; Wilson and Laurent, 2005; Wilson et al., 2004). Beside GABA also glutamate plays a crucial role as an inhibitory transmitter of the *Drosophila* AL, and is mediated by glutamate-gated chloride channel (GluClα) (Liu and Wilson, 2013).

In order to elucidate the source of the observed mixture inhibition, we used a pharmacological treatment, as a first approach, to block GABAergic and/or glutamatergic receptors while we monitored the odor-induced calcium signals in PNs. We therefore used picrotoxin (100 µM) to block the *Resistant to dieldrin (Rdl)* subunit of the $GABA_A$ receptor and the *GluClα*, we applied CGP54626 (50 µM) to selectively silence the $GABA_B$ receptor and we tested the mixture of both antagonists. By blocking the $GABA_B$ receptor type (Figure 5A), we noticed a reduction in the mixture inhibition in the four *attractive* glomeruli DM1, DM2, DM3 and DM4 compared to control (saline) or wash-out situation (Figure 5B and S3A). To quantify the amount of this reduction in the inhibition, we calculated the differences between the peak responses of the four glomeruli upon stimulation with the mixture and with the ethyl acetate stimulation alone, and normalized it to the highest average within a glomerulus. Hence, a value of 1 means strong mixture inhibition, while a value close to 0 represents no inhibition, i.e. the glomeruli responded equally to the mixture and the odor ethyl acetate. As expected, the peak response differences in the four *attractive* glomeruli were significantly reduced after CGP54626 treatment compared to the pre-treatment (saline) and wash-out (Figure 5C). Interestingly, after blocking the $GABA_A$ and glutamate receptors (Figure 5D), the two glomeruli DM1 and DM4 out of the four *attractive* glomeruli showed a significant reduction in their inhibition towards the mixture compared to ethyl acetate (Figure 5E; 5F and S3A). Notably, the mixture inhibition was totally abolished following the treatment with the two antagonists together (Figure 5G-5I and S3A). After applying picrotoxin at 100 µM concentration, the fly, did not survive the wash or the drug cannot be washed off as reported previously (Sachse and Galizia, 2002). The drugs had no obvious effect on the two major *aversive* glomeruli DL1 and DL5 on their responses to BEA and the mixture (Figure S3B) compared to the saline or the wash-out. It is know

that picrotoxin or CGP54626 treatments increase PNs responses to single odors (Root et al., 2008; Wilson and Laurent, 2005). However, we noticed that the drugs treatments increased the responses only in some of the glomeruli when stimulated with the attractive odor (ethal acetate 10^{-3}), but never reached to the threshold of (10^{-2}) activation, except for a few animals (compare signals in Figure 5 and 2E). After picrotoxin treatment, the responses of different glomeruli to the mixtures and the single odorants were faster than during the saline treatment, while the odor-evoked responses under CGP54626 treatment revealed a longer duration (see time traces in figure S4A and S4C). Picrotoxin was shown to affect the early response to odors while CGP54626 influences rather the late response (Wilson and Laurent, 2005).

Our pharmacological approach has two weak points: first, picrotoxin at the used concentration of 100 µM does not act specifically and blocks both, the *Rdl* subunit of the $GABA_A$ receptor as well as the *GluClα*. Second, the antagonists act on the pre- and postsynaptic sites and do not allow us to pinpoint where the inhibitaion takes place. To overcome these issues, we used RNA interference to specifically target GABAergic or glutamatergic receptors selectively at their pre- and postsynaptic sites in AL input and output neurons. We employed *UAS-Rdl RNAi* against the *Rdl* subunit of $GABA_A$ (Das et al., 2011; Liu et al., 2009), *UAS-GBi* against the *$GABA_BR2$* subunit (Root et al., 2008), *UAS-gluclα RNAi* against the *GluClα* (Liu and Wilson, 2013) and *UAS-empty-RNAi* as a control. We confirmed the efficiency of the RNAi lines by immunostaining and RT-PCR (Figure S4A).

First, we targeted those RNAi lines individually on the postsynaptic sites (i.e. PNs) while visualizing the odor-evoked calcium signals from PNs after stimulation with the repellent odor benzaldehyde (10^{-1}), the attractant odor ethyl acetate (10^{-2} or 10^{-3}) and the binary mixtures (MIX (-)) (Figure 6A “upper panel”). Interestingly, blocking $GABA_A$ receptors selectively in PNs significantly reduced the inhibition to the mixture in two glomeruli (DM1 and DM4) compared to the control, while the inhibition in the other two *attractive* glomeruli (DM2 and DM3) was unchanged (Figure 6A “lower panel”, 6B). Silencing $GABA_B$-receptors and *GluClα* in PNs did not affect the observed mixture inhibition in all *attractive* glomeruli (Figure 6A “lower panel”, 6B). The *aversive* glomeruli (DL1 and DL5)

revealed a linear mixture response independent of the RNAi line measured (Figure S4B). In addition, we did not observe any effect of the different RNAi regarding the higher mixture concentration (i.e. benzaldehyde 10^{-1}, ethyl acetate 10^{-2} and MIX (+)) (Figure S4C). These findings indicate that the $GABA_A$ receptor mediates the inhibition to the mixture on the postsynaptic sites (PNs) in two out of the four *attractive* glomeruli. These results are consistent with our findings deriving from the pharmacological treatments, showing that the $GABA_A$ antagonist leads to a suppression of the inhibition glomeruli of DM1 and DM4 only (Figure 5E and 5F).

From our pharmacological experiments, application of CGP54626 ($GABA_B$ receptors antagonist) significantly reduced the inhibition in all of the four glomeruli to the mixture (Figure 5A and 5B). However, we did not observe the same effect by silencing the $GABA_B$ receptors using the RNAi approach targeting selectively PNs. The $GABA_B$-mediated inhibition on the presynaptic sites (ORNs) has been already well characterized (Olsen and Wilson, 2008; Root et al., 2008). To investigate whether part of the observed mixture inhibition of the *attractive* glomeruli occurs on the presynaptic site and is mediated by the $GABA_B$ receptor, we sought to selectively silence the GABAergic and glutamatergic receptors in ORNs while monitoring the calcium responses to the mixtures in PNs. By silencing only $GABA_B$ receptor in ORNs, the mixture inhibition was abolished in glomeruli DM2 and DM3 and strongly suppressed in glomeruli DM1 and DM4 (Figure 6C “lower panel” and 6D). We did not observe any effects on the odor-evoked responses in the *aversive* glomeruli nor at high odor concentrations by any of the RNAi lines (Figure S4D and S4E).

Since our results show that the $GABA_B$ receptor mediates the mixture inhibition on the presynaptic sites in ORNs, we wondered whether we can observe the mixture inhibition on ORNs. To analyze that, we performed calcium imaging in ORNs in the AL using the same odors: benzaldehyde (10^{-1}), ethyl acetate (10^{-2} and 10^{-3}), and their binary mixtures. Both *attractive* and *aversive* glomeruli responded to the high concentration mixture as predicted, i.e. in the same as they responded to the corresponding individual odors (Figure S5A). However, the *attractive* glomeruli were inhibited by the low

370 concentration mixture (MIX (-)), two of which (DM2 and DM3) were strongly inhibited,
371 while DM1 and DM4 were only mildly inhibited (Figure S5B).

372 The basiconic sensillum ab1 housed both, ORNs responding to ETA (i.e. Or42b
373 innervating glomerulus DM1) and BEA (i.e. Or10a targeting glomerulus DL1) (Clyne et
374 al., 1999; Couto et al., 2005; Gao and Chess, 1999; Gao et al., 2000; Hallem and
375 Carlson, 2006; Hallem et al., 2004; Vosshall, 2000; Vosshall et al., 1999). To check
376 whether non-synaptic inhibition between those ORN types housed in the same
377 sensillum might have any influence onto the observed mixture inhibitions (Su et al.,
378 2012), we performed single sensillum recordings (SSR) from the ab1 sensillum.
379 However, we did not find any differences between the response to ETA (10^{-3}) and the
380 low concentration mixture, as well as between ETA 10^{-2} and the high concentration
381 mixture (Figure S5C). These data show that the inhibition of the *attractive* glomeruli to
382 the low concentration mixture derives from the AL network at the ORN level.

383 In sum, these results altogether show that mixture inhibition in the attractive channels to
384 a binary mixture of opposite valences is mediated by GABA. Interestingly, the *attractive*
385 glomeruli act differently on the inhibition. Glomeruli DM2 and DM3 were inhibited on the
386 presynaptic locus through the $GABA_B$ receptor, while glomeruli DM1 and DM4 were
387 inhibited on the pre- and postsynaptic terminals via $GABA_B$ and $GABA_A$ receptors,
388 respectively. To the best of our knowledge, our results reveal for the first time that some
389 glomeruli are inhibited by both $GABA_A$/$GABA_B$ on two different loci (pre- and
390 postsynaptic), while other glomeruli are inhibited solely on the presynaptic site. This
391 might explain the difference in the sensitivity between different glomeruli (Hong and
392 Wilson, 2015).

393

394 **Defined subset of GABAergic LNs mediates glomerulus-specific inhibition**

395 Our results suggest that GABAergic LNs within the AL serve as the key factor of the
396 mixture inhibition to odors with opposing behavioral values. To verify that inhibitory LNs
397 mediate the inhibition at the PN level, we selectively silenced the GABA release of
398 different inhibitory LN populations using four different enhancer trap lines and monitored

399 the calcium responses to the mixture and the individual odor components in PNs (Fig.
400 XX). We blocked the GABA release by knocking down glutamic acid decarboxylase
401 (GAD), an important enzyme for the GABA synthesis (Jackson et al., 1990; Küppers et
402 al., 2003) using *Gad-RNAi.* The used Gal4 lines in this study label different types of
403 GABAergic LNs ranging from pan-glomerular, continuous, regional, to patchy. GH298-
404 Gal4 labels mostly pan-glomerular LNs, NP3056-Gal4 labels combination of pan-
405 glomerular and patchy LNs, H24 and HB4-93 Gal4 lines label mostly regional and
406 patchy LNs (Chou et al., 2010; Thum et al., 2011). We performed immunohistochimstry
407 to confirm the efficiency of *UAS-GAD RNAi* (Figure 6A-6D???). Surprisingly, silencing
408 GABA synthesis in different subsets of LNs labelled by different Gal4 lines had different
409 effects on the mixture inhibition in the different *attractive* glomeruli. Mixture inhibition in
410 DM3 was abolished by silencing GABAergic LNs in NP3056-Gal4 (Figure 6B', 6B''').
411 Silencing GABA in the LNs of HB4-93 Gal4 abolished mixture inhibition in DM1 and
412 DM4 (Figure 6D', 6D''). GABAergic LNs in GH298 and H24 Gal4 lines did not have a
413 major role in the mixture inhibition in the four *attractive* glomeruli (Figure 6A', 6A'' and
414 6C', 6C'', respectively). Interestingly, we observed an increase in the mixture inhibition
415 in some of the attractive glomeruli upon silencing GABAergic LNs of some Gal4 (Figure
416 6A'' and 6B''').

417

418 **Specific cross-talk between *aversive* and *attractive* glomeruli.**

419 Previous studies suggested that inhibition in the AL can be sparse and specific, pan-
420 glomerular and global, or even both (Asahina et al., 2009; Girardin et al., 2013; Ng et
421 al., 2002; Olsen et al., 2010; Sachse and Galizia, 2002; Silbering and Galizia, 2007;
422 Silbering et al., 2008). Furthermore, it has been shown that certain glomeruli exhibit
423 different sensitivities towards an inhibitory input (Hong and Wilson, 2015). Specific
424 inhibition and glomerulus-specific sensitivity to inhibition suggests a specific cross-talk
425 between glomeruli achieved by the interneuron network. Since our results demonstrate
426 that binary mixtures induce glomerulus-specific inhibitions mediated by iLNs at both, the
427 pre- and postsynaptic level, we postulate a glomerulus-specific interaction between
428 aversive and attractive glomeruli in the context of binary mixture of opposing valence.

To test this hypothesis, we used two approaches: First, we investigated at a functional and behavioral level the effect of selectively silencing the input to the aversive glomeruli DL1 or DL5. Second, to test for sufficiency, we asked what happens if we replace the aversive odor by optogenetically activating the aversive glomeruli, while presenting the attractive odor?

To do so, we first monitored the calcium signals from PNs after stimulation with the binary mixture of benzaldehyde and ethyl acetate and the single odors in flies where the benzaldehyde-responsive glomeruli, DL1 or DL5, was selectively silenced using a mutant background of Or10a or Or7a, respectively (de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004) (Figure 7A and 7D). Both mutants reveal no odor-evoked PN activity in the corresponding glomerulus (Figure 7B, 7C, E, 7F, S6A and S6B), indicating that lateral excitatory seems not to take place in these cases (Badel et al., 2016; Olsen et al., 2007; Root et al., 2007; Shang et al., 2007).Stimulation with MIX (+) represents the linear combination of both individual odor and its single odors (BEA 10^{-1} and ETA 10^{-2}) in flies bearing the Or10a mutant background (Figure S6A). However, the mixture inhibition in the two *attractive* glomeruli DM1 and DM4 to the low concentration mixture (MIX (-)) was, interestingly, abolished, while the inhibition of the other two *attractive* glomeruli DM2 and DM3 was still visible (Figure 7B and 7C). On the other hand, flies bearing an Or7a mutation showed normal mixture inhibition in DM1 and DM4 to stimulation to MIX (-) (Figure 7E and 7F). However, surprisingly, the inhibition to the MIX (-) in DM3 was abolished (Figure 7E and 7F) and was reduced in glomerulus DM2 compared to the control flies (compare signals in Figure 7F and 2E). We did not observe no obvious effect of the Or7a mutation on the activity of the *attractive* and *aversive* glomeruli when the high concentration mixture (MIX (+)) was applied (Figure S7B).

We next asked whether the modification of the inhibitory pattern regarding the attractive glomeruli caused by silencing one of the aversive receptors (Or10a or Or7a) would also be reflected by the behavioral output of the fly. We therefore turned to a two-choice bioassays in order to quantify the behavioral effect of the mixture. Using two control lines (wild type CS and *w1118*), benzaldehyde at 10^{-1} concentration was highly aversive

459 to the flies, while ethyl acetate at both concentrations (10^{-2} and 10^{-3}) were highly
460 attractive compared to the solvent mineral oil (MOL). MIX (+) induced strong attraction
461 in control flies, while MIX (-) failed to attract flies (Figure 7G). The Or10a mutant flies
462 showed the same behavior as the control flies except for MIX (-), where they were
463 significantly more attracted to the aforementioned mixture as the control flies (Figure
464 7G). Strikingly to us, the Or7a mutant flies behaved exactly the same as the control flies
465 towards the mixtures and the single odors (Figure 7G). Notably, the aversive effect of
466 benzaldehyde regarding the Or10a and Or7a mutant flies was slightly reduced
467 compared to the control flies. To assess whether the attraction to MIX (-) in the Or10a
468 mutant flies was a result of the reduction of the aversion to benzaldehyde or the
469 abolishing of the inhibition in glomeruli DM1 and DM4, we modified our T-maze assay in
470 a way that compare flies' attraction to the binary mixtures against their corresponding
471 concentration of ethyl acetate. When testing MIX (-) against ethyl acetate at (10^{-3}), the
472 flies showed a stronger preference for the attractive odor ETA. However, the aversion to
473 MIX (-) was significantly reduced in the Or10a mutant flies with no obvious differences
474 in the behavior of the Or10a mutant flies and the control ones to MIX (+) (Figure 7E).
475 These results indicate that the attraction to MIX (-) (Figure 7D) is caused by the ablation
476 of the inhibition of glomeruli DM1 and DM4, and not because of the reduction in the
477 aversion to BEA. Notably, Or10a and Or7a mutant showed strong aversion towards
478 benzaldehyde (Figure 7G), indicating that aversion to benzaldehyde is mediated by a
479 combination of several glomeruli including the strongest activated glomeruli DL1 and
480 DL5 and other minor activated glomeruli.

481 In sum, our results show that the *aversive* glomerulus DL1, which is innervated by
482 Or10a-expressing ORNs, is mediating the inhibition of the two *attractive* glomeruli DM1
483 and DM4 upon stimulation with MIX (-). Moreover, by blocking the inhibition in DM1 and
484 DM4 by silencing Or10a, flies were attracted to MIX (-). On the other hand, we assume
485 that glomerulus DM3 and, to some extent, DM2 are inhibited by the *aversive* glomerulus
486 DL5 (innervated by Or7a-expressing neurons), although we could not observe any
487 changes in the behavior of flies in which Or7a was mutated. One possible interpretation,
488 among others, is that the inhibition of DM2 and DM3 glomeruli affects other behavioral
489 parameters that we could not capture by our behavioral assay.

Discussion

Evaluating olfactory stimuli in early sensory processing is a crucial step for signal recognition and to execute the right instinctive behavior. However, the majority of olfactory stimuli represent complex mixtures. In this study, we analyzed the mixture interaction of two odorants of opposing hedonic valences, and demonstrate how glomerular-specific inhibition and crosstalk results in an appropriate behavioral output.

Interglomerular mixture inhibition

Mixture interactions have been well studied in vertebrates (Gupta and Stopfer, 2014; Howard and Gottfried, 2014; Saraiva et al., 2016; Tabor et al., 2004) and invertebrates (Fujiwara et al., 2014; Olsen et al., 2010; Riffell et al., 2009a; Riffell et al., 2009b; Silbering and Galizia, 2007; Su et al., 2011; Su et al., 2012; Thoma et al., 2014); however, the origin and the underlying neuronal mechanisms of mixture interaction have still remained elusive.

Flies use several strategies to navigate towards an odor source. It has been shown that vinegar flies tend to walk or fly upwind in response to odors (Becher et al., 2010; Bhandawat et al., 2010; Budick and Dickinson, 2006; Knaden et al., 2012; Semmelhack and Wang, 2009; Steck et al., 2012; Thoma et al., 2014). Here, we chose a walking behavioral paradigm (FlyWalk) to monitor the fly's behavior. Consistent with a previous study (Thoma et al., 2014), our data show that adding an aversive odor to an attractive odor in a binary mixture suppresses the attractiveness of the attractive odor. However, we did not observe similar results when a higher concentration of the attractive odor was used. Our results on the olfactory behavioral output of the flies upon facing a mixture of opposing valence are comparable to the feeding behavior of flies in similar situations. Sugar and bitter sensitivities of *Drosophila* change to mixtures with different concentrations of sugar and bitter compounds and according to their internal status which are modulated by neuromodulatory pathways (Inagaki et al., 2014).

Using two-photon functional imaging to monitor the activity of postsynaptic PNs in the ALs of different glomeruli (using GH146-Gal4) we were able to annotate most glomeruli from the 5-6 focal planes we imaged from. We compared the signal intensities from different glomeruli upon stimulation with the binary mixtures and their corresponding mixture components. Representations of binary mixtures can be either explained via elemental coding (i.e. the summation of the activated glomeruli by each of the single mixture constituent) or via configural coding (i.e. recruitment of an additional network) (Honey et al., 2014; Howard and Gottfried, 2014; Linster and Cleland, 2004; Melchers et al., 2008; Thomas-Danguin et al., 2014). As expected, we observed that those glomeruli that were activated by the mixture were the same that were activated by the single components of the mixture with no recruitment of any additional glomeruli (Figure S2). However, we noticed a clear and strong inhibition of those glomeruli that responded to the attractive odor (ethyl acetate) when it was presented at a low concentration in a mixture with an aversive odor (benzaldehyde). Notably, those glomeruli that were activated by the aversive odor did not show any mixture interaction and revealed the same level of activation to the mixture and the individual odors. We assume that the observed glomerulus-specific mixture inhibition might be due to the heterogeneity of the inhibition strength between different glomeruli (Hong and Wilson, 2015). Heterogeneity in responses to mixtures has been shown in previous studies where excitation of some glomeruli by one of the mixture components can inhibit the glomeruli activated by the other component (Silbering and Galizia, 2007; Tabor et al., 2004), or mixtures can produce sublinear summation (Olsen et al., 2010). We did not observe this inhibition when we used a higher concentration of the attractive odor (ethyl acetate) combined with the same concentration of the aversive odor (benzaldehyde), the same mixture concentrations that showed no suppression in the attractiveness towards ethyl acetate behaviorally. One possible explanation could be, that the OSN-PN synapses are non-linear (Kazama and Wilson, 2008) due to the saturation of the vesicular release at high presynaptic firing rates. Hence, lateral inhibition deriving from the activated aversive circuit could not decrease the probability of vesicular release at high activation of *attractive* glomeruli (i.e. ETA 10^{-2}) (Olsen and Wilson, 2008). Conversely, we did not observe any inhibition in the attractive glomeruli upon activation with the aversive odor.

549 This is likely due to the low spontaneous activity of the cognate ORNs activated by ETA
550 (Hallem and Carlson, 2006), since spontaneously spike activity of PNs is mainly due to
551 spontaneous activities of cognate ORNs that produce a correlated spontaneous
552 fluctuations in the membrane potential of the postsynaptic PNs (Bhandawat et al., 2007;
553 Gouwens and Wilson, 2009; Kazama and Wilson, 2009; Schlief and Wilson, 2007;
554 Silbering et al., 2008; Wang et al., 2003) .

555 The strength of activation of ORNs will be conveyed to the activation of PNs and will
556 dependently recruit lateral inhibition differently (Bhandawat et al., 2007; Hong and
557 Wilson, 2015; Seki et al., 2017; Wilson and Laurent, 2005; Wilson et al., 2004). By
558 lowering the activation of aversive circuits by using a lower concentration of the aversive
559 odor (BEA 10^{-2}), we have not observed the behaviorally and physiologically drop in the
560 flies' responses upon stimulation with a mixture of the low concentration of the attractive
561 odor (ETA 10^{-3}) and a lower concentration of the aversive odor (BEA 10^{-2}) compared
562 with their responses to a stimulation with the attractive odor alone. Thus, the
563 suppression in behavior and physiology was restored upon lowering the concentration
564 in the attractive odor to (10^{-4}) in the mixture with BEA 10^{-2} in comparison to the flies'
565 responses to ETA 10^{-4}. Most of the previous studies on mixture interactions were limited
566 to only a single concentration of the odors in a mixture (Carlsson et al., 2007; Deisig et
567 al., 2006; Duchamp-Viret et al., 2003; Gupta and Stopfer, 2014; Silbering and Galizia,
568 2007; Tabor et al., 2004). However, our results argue that interglomerular inhibition in
569 response to a mixture of attractive and aversive odors depends on the level and the
570 ratio of activation of the two subsets of ORN-types activated by the two odors (i.e. the
571 ratio between the two odors presented). Previous work on vertebrates' mitral/tufted cells
572 of the olfactory bulb and invertebrates' PNs of the AL showed either linear (Badel et al.,
573 2016; Davison and Katz, 2007; Gupta and Stopfer, 2014; Khan et al., 2008) or non-
574 linear mixtures interactions (Davison and Katz, 2007; Giraudet et al., 2002; Niessing
575 and Friedrich, 2010; Shen et al., 2013; Tabor et al., 2004). Here, we demonstrate that
576 the behavioral output reflects the gradual non-linear activity of PNs.

577 Our data shows that by changing the subsets of ORN-types activated by the attractive
578 and aversive odors in binary mixtures, different inhibitory patterns arise which correlate

579 to the behavioral output (i.e. less attraction to the mixture compared to the attractive
580 odor). Pairing balsamic vinegar with benzaldehyde shows a reduction in the behavioral
581 attractiveness. At the functional level, physiological inhibition was observed in four out
582 of seven glomeruli which were highly activated by balsamic vinegar. This result can be
583 interpreted that the other three glomeruli do not contribute to the balsamic vinegar
584 attractiveness, in which accumulating evidence suggests that the innate behavioral
585 output is correlated to either the summed weights of some of the glomeruli activated or
586 to the activity of single processing channels (Ai et al., 2010; Badel et al., 2016; Dweck et
587 al., 2013; Knaden et al., 2012; Min et al., 2013; Parnas et al., 2013; Ronderos et al.,
588 2014; Semmelhack and Wang, 2009; Wilson, 2013). One single exception in our data
589 set showed that the usual mixture effect on the behavioral output (geosmin and
590 balsamic vinegar), was not correlated with any inhibition of attractive glomeruli
591 (activated by balsamic vinegar) at the PN level (Figure 4D-4D'''). Geosmin activates a
592 single dedicated channel, so-called a labeled-line, which is ORNs expressing the Or56d
593 receptor and innervating glomerulus DA2 in the AL (Couto et al., 2005; Stensmyr et al.,
594 2012). The DA2 glomerulus is innervated by a large number of PNs and has a relatively
595 low LN innervation density (Grabe et al., 2016), which might propose that this particular
596 glomerulus does not have any strong interglomerular interactions with other glomeruli.
597 One brain region where the mixture interaction between GEO and BAL might be
598 implemented is the LH which contains circuit elements that have been shown to
599 mediate interactions between odors (Fisek and Wilson, 2014; Jefferis et al., 2007; Lai et
600 al., 2008; Liang et al., 2013; Owald et al., 2015; Parnas et al., 2013; Seki et al., 2017;
601 Strutz et al., 2014). Another higher brain region that can accommodate this mixture
602 interaction could be the MB, where a previous study showed that MB output neurons
603 (MBONs) are responsible for a mixture interaction of vinegar and CO_2; notably CO_2
604 represents another labeled line, similarly structured as the geosmin circuitry.

605

606 **GABAergic inhibition is crucial for mixture integration**

607 Lateral inhibition in the *Drosophila* AL is mainly mediated through the neurotransmitter
608 GABA (Ng et al., 2002; Wilson and Laurent, 2005; Wilson et al., 2004), while glutamate

609 (Liu and Wilson, 2013), tachykinin (Ignell et al., 2009) or some other neuropeptides
610 might contribute as well (Carlsson et al., 2010). Among several functions of this
611 GABAergic inhibition, the main one represents the gain control phenomenon (Olsen and
612 Wilson, 2008; Root et al., 2008). In addition it leads to more transient PN responses
613 (Olsen et al., 2010) and coordinates synchronous oscillations among PNs (Tanaka et
614 al., 2009), while it was shown in the same study that this oscillation has only a small
615 contribution to olfactory processing in *Drosophila*. Moreover, GABAergic inhibition also
616 contributes in the mixture interaction between two odors (Olsen et al., 2010; Silbering
617 and Galizia, 2007) which is in line with our study.

618 Most of the lateral GABAergic inhibition has been shown to take place predominantly on
619 the presynaptic site, i.e. at the ORN axon terminals mediated through $GABA_A$ and
620 $GABA_B$ receptors (Nagel et al., 2015; Olsen and Wilson, 2008; Root et al., 2008). Most
621 lateral inhibition vanishes, when ORNs have been silenced (Olsen and Wilson, 2008).
622 Moreover, ORN axon terminals are positive immunoreactive towards GABA receptors
623 (Root et al., 2008). However, PNs also receive GABAergic inhibition via $GABA_A$ and
624 $GABA_B$ receptors from LNs (Wilson and Laurent, 2005).

625 It was shown that the ionotropic $GABA_A$ receptors mediate the inhibition in early stages
626 of the odor-evoked responses, while the metabotropic $GABA_B$ receptors act on the late
627 phases of the responses (Olsen and Wilson, 2008; Wilson and Laurent, 2005). Lateral
628 inhibition caused by mixture interaction happens either in the periphery between two
629 neurons housed in the same sensillum (Su et al., 2011; Su et al., 2012), or at the
630 synaptic level in the AL which can be blocked with a combination of $GABA_A$ and $GABA_B$
631 receptors antagonists (Olsen et al., 2010; Silbering and Galizia, 2007), or a global
632 inhibition and glomerulus-specific lateral excitation (Olsen et al., 2007). Here, we show
633 that the aversive odor inhibits the attractive odor channels in different patterns.
634 Strikingly, we found that two out of the four *attractive* glomeruli (DM1 and DM4) are
635 inhibited on both synaptic levels (i.e. presynaptic on the ORNs axon terminals via
636 $GABA_B$-receptors and postsynaptic on the PNs via $GABA_A$-receptors), while the other
637 two *attractive* glomeruli (DM3 and DM4) are inhibited predominantly on the presynaptic
638 level through $GABA_B$-type receptors. GABAergic receptors at the PNs level are so far

639 not well studied and it was assumed that they contribute weakly to the inhibition (Wilson
640 and Laurent, 2005). In this study we show that $GABA_A$-type receptors have a major role
641 on the inhibitory mixture interaction on the PNs level in, at least, two glomeruli.

642 Why do different glomeruli undergo different inhibition strategies? The answer to this
643 question needs to be elucidated with further studies. Nonetheless, some evidence
644 showed that different glomeruli show different degrees of sensitivity to lateral inhibition.
645 One study reported that some glomeruli are strongly inhibited by lateral inhibition (such
646 as DL2) while others (such as DC4) are weakly inhibited, even though both glomeruli
647 are activated by the same odorant and have the same amount of GABA release (Hong
648 and Wilson, 2015). Other study demonstrated that a one glomerulus was systematically
649 more susceptible to mixture suppression than another glomerulus, although both
650 glomeruli were inhibited by the same component of the mixture (Olsen et al., 2010). All
651 this spatial inhomogeneity of sensitivity of lateral inhibition may result in different
652 glomeruli bearing different inhibition strategies in different situations, either presynaptic
653 inhibition, postsynaptic inhibition, or both.

654 What might be the role of LNs in this mixture inhibition? Whether LN activity is global or
655 specific has been debatable. The morphology of LNs is consistent with both theories.
656 Most individual LNs innervate most or all glomeruli, which could lead to a global
657 inhibition within the AL. However, some LNs innervate small subsets of glomeruli (e.g.
658 patchy or regional LNs) and could permit specific interactions between different
659 glomeruli (Chou et al., 2010; Das et al., 2008; Lai et al., 2008; Okada et al., 2009; Seki
660 et al., 2010; Shang et al., 2007; Stocker et al., 1990; Wilson and Laurent, 2005). One
661 recent study showed that the activity of LNs is global and that GABA release equals in
662 most of the glomeruli (Hong and Wilson, 2015). Another study, using a fluorescent
663 sensor of vesicular release to measure GABA release in different glomeruli, showed a
664 specific GABA release in different glomeruli which dependent on the odor stimulus (Ng
665 et al., 2002). However, the former study used two Gal4 enhancer trap lines which
666 mostly label pan-glomerular LNs. Hence they might have missed the effect of the patchy
667 or regional LNs (Chou et al., 2010). The later study used a GAD1-Gal4 line that covers
668 the whole GABAergic LNs in the AL. Our results show that the lateral inhibition in the

669 *attractive* glomeruli caused by the odor mixture disappeared when we silenced GABA
670 synthesis by using RNAi against *GAD1* in defined subsets of different LNs labeled by
671 NP3056-Gal4 and HB4-93-Gal4 which label mostly patchy LNs (Chou et al., 2010). Of
672 course, global inhibition caused by global release of GABA occurs in the AL and most
673 probably results in gain control.

674 Our results, simply, show that stimulating the olfactory system of *Drosophila* with a
675 mixture of attractive and aversive odors results in inhibition in the *attractive* glomeruli
676 which is caused by two major factors: a) the sensitivity of different glomeruli to the
677 lateral inhibition, and b) the recruitment of selective inhibitory LNs. Regarding the
678 heterogeneity of different glomeruli towards sensitivity to inhibition, we see that
679 phenomenon in the different inhibition levels of the *attractive* glomeruli in response to
680 different excitation levels of the attractive and aversive circuits using different
681 concentrations of the two components of the mixtures. In addition, the mixture inhibition
682 is mediated by the recruitment of specific GABAergic LNs which seem to be activated
683 by the simultaneous activation of both, attractive and aversive channels.

684

685 **Specific glomerular crosstalk**

686 In other neuronal circuits, some neurons receive inhibition selectively from specific
687 inhibitory neurons (Briggman et al., 2011; Gibson et al., 1999; Yoshimura and Callaway,
688 2005). In the *Drosophila* AL, two studies suggested two separate models in which
689 specific subsets of glomeruli are connected with inhibitory LNs and activation of specific
690 ORNs recruits input to other specific glomeruli (Ng et al., 2002; Silbering and Galizia,
691 2007). However, these studies did not show the neuronal mechanism behind their
692 models. Our data, indeed, support the aforementioned studies with regard to specific
693 lateral inhibition between subsets of glomeruli. Using genetic manipulations (silencing
694 and activation) of ORNs activated by aversive odors (Or10a and Or7a innervating DL1
695 and DL5, respectively) while having the mixture inhibition as a physiological output, we
696 were able to show that the aversive circuits (DL1 and DL5) are linked via inhibitory
697 subnetworks to the attractive channels (DM1, DM2, DM3 and DM4). The DL1 circuit

inhibits the DM1 and DM4 channels, while DL5 suppresses DM3 and, to some extent, DM2 (Figure 8 the model).

Interestingly, manipulation of one aversive channel (i.e. DL1), which in turn is linked to DM1 and DM4, showed impairment in the behavioral output of the flies facing MIX (-), which shows the importance of DM1 and DM4 in the attraction behavior. This finding is in line with previous studies that show the significant role of Or42b and Or59b (DM1 and DM4, respectively) in flies attraction towards odors (Knaden et al., 2012; Semmelhack and Wang, 2009).

In sum, our results show that flies react differently in response to binary mixtures of different concentrations of attractive and aversive odors. These behavioral outputs to mixtures are not concentration-dependent, but rather ratio-dependent between the two components of the mixture. Our results also suggest that mixture interactions happen in early stages of olfactory processing in the *Drosophila's* olfactory system (i.e. the AL), where the attractive circuits are inhibited by the aversive channels. This inhibition is a result of the neurotransmitter GABA, in which the level on which GABA acts varies in different glomeruli. Our data illustrate that there are some factors which influence the lateral inhibition, namely, the level of excitation of ORN-PN in a specific glomerulus, the sensitivity of different glomeruli to the inhibition, and the recruitment of a specific inhibitory network when both channels (attractive and aversive) are activated. Finally, the notion of specific inhibitory interactions between specific subsets of glomeruli has been disputable for a long time. Here, we show, for at least our data set that some of the known *aversive* glomeruli are linked with and therefore crosstalk to some of the *attractive* glomeruli, thereby providing evidence that specific lateral inhibition occurs in the Drosophila AL (Figure 8 the model).

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Material and Methods

Fly stocks

Flies were reared on conventional cornmeal agar medium under 12h/12h light/dark cycle at 25^{o}C (except for the experiment involving PCR, which were reared at 18^{o}C). All experiments were performed on adult females. The following stocks were used: Canton-S wildtype flies, GH146-Gal4 (Stocker et al., 1997) (from Leslie Vosshall's lab.), 20XUAS-IVS-GCaMP6s (*attP40*) (Chen et al., 2013) (Ilona Kadow's lab.), 20XUAS-IVS-GCaMP6f (VK00005) (Bloomington stock center (BL) 52869), GH298-Gal4 (Stocker et al., 1997) (Stocker's lab.), NP3056-Gal4 (*Drosophila* Genomics Resource Center DGRC113080) (Hayashi et al., 2002), HB4-93-Gal4 (liqun luo's lab.), H24-Gal4 (BL51632), Or10a mutant (Or10a[f03694]) (Thibault et al., 2004) (BL 18684), Or7a mutant (Or7a$^{-/-}$) (Lin et al., 2015) (gift from Christopher Potter), UAS-Rdli-G and UAS-GBi (Liu et al., 2007 and Root et al., 2008, respectively) (both are gift from Mani Ramaswami), UAS-dicer2 (Liu and Wilson 2013) (BL24644), nsyb-Gal4 (BL51635), Gal80^{ts},(BL7018) UAS-GluClα RNAi (Liu and Wilson 2013) (Vienna *Drosophila* RNAi Center VDRC 105754). Or10a-Gal4 (Vosshall et al., 2000) (BL9944), Or7a-Gal4 (Vosshall et al., 2000) (BL23908), UAS-empty RNAi (*attp*40) (BL36304), 20x UAS-CsChrimson-mCherry-trafficked (*VK00005*) and 20x UAS-CsChrimson-mCherry-trafficked (*in su(Hw)attP5*) (both are gifts from Vivek Jayaraman), Orco-Gal4 (gift from Andrea Fiala), UAS-GAD1 RNAi (Ni et al., 2008) (VDRC 32344) and GH146-QF,QUAS-GCaMP3.0 (gift from Kazama's lab.).

Flywalk

Flywalk experiments were conducted as described before in (Thoma et al.). In brief, we tested 15 female flies in 15 parallel glass tubes (inner diameter 0.8 cm). The flies were continuously exposed to a humidified airstream with a velocity of 20 cm/s (20 °C, 70 % relative humidity). All experiments were performed under red light background conditions (λ = 630 nm) made by a LED cluster. Flies were monitored during the whole experiment using an overhead camera (HD Webcam C615, Logitech, Switzerland). We

presented odors using a multicomponent stimulus device, where flies were repeatedly presented 1 s pulses of single odors or mixtures with an interstimulus interval of 90 s. At the same time the flies´ XY-position was recorded for each pulse. The stimulus protocol consisted of 2 single odors, their mixture, a negative control (mineral oil, MOL) and clean air pulses, which were presented for 50 times each in a pseudorandomized sequence.

Odors were presented *via* small vials made of polyetheretherketone (PEEK) containing 100 µl of odor dilution in a 0.2 ml PCR reaction tube. The vials were tightly sealed using a rubber seals and connected to the mixing chamber of the Flywalk´ odor delivery system *via* ball-stop check valves. The mixing chamber itself was also made of PEEK and had connections with 8 circularly arranged 3-way solenoid valves passing 8 different airflows into the chamber. Basically there are two airflows – continuous and odor. The continuous airflow stands for the “no odor”-condition, where the clean airflow passes through empty and clean odor vials. While presenting an odor, the clean airflow is redirected through the vial containing the odor dilution, picking up the saturated headspace. Thus, odors are presented to the flies with minimal disturbances in the total airflow.

Flywalk analysis

Since flies are allowed to move freely in the glass tubes, the individuals may have different meeting times with the same odor pulse, depending on whether they sit more upwind or downwind. We corrected this by calculating the encounter for each single fly for each stimulus based on its position, the delay of the odor travelling through the system and the wind speed within the system using a custom-written script in R (https://www.r-project.org/). A second custom-written script was used to calculate the response of the flies towards an odor. On one hand we calculated the mean movement speed of the flies from 1 s before the odor until 7 s after the odor pulse (Figure 1B and 1E). Therefore we calculated first the average speed within each fly and in the next step we calculated the mean of all flies from the individual averages. When analyzing the

upwind displacement (Figure 1D and 1G) (the distance the flies walked upwind after the odor pulse) we used the same approach, but only within 4 s after the odor pulse.

Two-Photon calcium imaging

All calcium imaging experiments were performed on starved (24h) female flies aged 4-6 days post-eclosion unless other mentioned.

The experimental preparation of the flies for calcium imaging was previously described in (Struze et al., 2012). In short, flies were briefly cold-anesthetized on ice and fixed with the neck onto a custom-made Plexiglas mounting block with copper plate (Athene Grids, Plano) and a needle before the head to stabilize the proboscis. Head was glued to the stage using Protemp II (3M ESPE) and the antennae were pulled forward by a fine metal wire. A small plastic plate with a hole that is covered with polyethylene foil was placed on the fly's head. A small cut in the foil was made to expose the head; the foil was sealed to the cuticle using two-component silicon (World Precision Instruments) to prevent the leaking of the Ringer's solution onto the antennae. We added Ringer's solution (NaCl: 130 mM, KCl: 5 mM, $MgCl_2$: 2 mM, $CaCl_2$: 2 mM, Sucrose: 36 mM, HEPES-NaOH (pH 7.3): 5 mM) (Estes et al., 1996) to the exposed head, and the head cuticle was removed. Care was taken while removing all fat, trachea, and air sacs to reduce light scattering. Antennal lobes were imaged from the dorsal side using 2-photon laser scanning microscope (2PCLSM, Zeiss LSM 710 meta NLO) equipped with an infrared Chameleon UltraTM diode-pumped laser (Coherent, Santa Clara, CA, USA) and a 40x water immersion objective lens (W Plan-Apochromat 40x/1.0 DIC M27), The microscope and the laser were placed on a smart table UT2 (New Corporation, Irvine, CA, USA). The fluorophore of GCaMP was excited with 925 nm. Fluorescence was collected with an internal GaAsP detector through a bandpass emission filter (BP470-550). For each individual measurement, a series of 40 frames acquired at a resolution of 256x256 pixels was taken with a frequency of 4 Hz. To cover the whole antennal lobes, we imaged from 5-6 imaging planes (depending on the preparation) which cover the dorsal-ventral axis of the antennal lobe at ~ 25-30 µm intervals in figure 2, 3, 4 and

S2. Furthermore, no significant odor-evoked signals were observed in between these imaging planes for any of the odors used. The rest of the experiments, we focused on the three focal plans where our main *attractive* and *aversive* glomeruli (DL1, DL5, DM1, DM2, DM3, and DM4) are accessible. In some cases where the glomeruli's boundaries were not easily detected, we acquired a high-resolution z-stack (1024x1024 pixels) at the end of the experiment.

For pharmacological experiments**,** antagonists were prepared in concentrated stock solutions in Dimethyl sulfoxide (DMSO, Sigma). Just prior to the experiments, we diluted the stock solution into Ringer's saline to obtain the final concentration. Picrotoxin (PTX) (Sigma) was prepared as 4.2 mM stock solution in DMSO, while 3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP54626) (Tocris) was prepared as 5.6 mM stock solution in DMSO. Both drugs were diluted in 500 µl saline, just prior to the experiment, to reach the final concentration.

Odor delivery system for calcium imaging experiments.

We have developed a computer-controlled odor delivery system that is similar to the one used in the Flywalk to a great extent (Figure 2A). Pure compounds were diluted in mineral oil (Carl Roth GmbH + Co. KG) and in water in case of balsamic vinegar. 2 ml of the diluted odors were added to glass bottle (50 ml, Duran Group, Mainz, Germany), with two sealed openings for the in-and-out of the air flow. For odor application, we used LabVIEW software (National Instruments Germany GmbH) which was connected to ZEN software (Zeiss) to triggers both image acquisition as well as odor delivery. A continuous airstream, whose flow of 1 l min^{-1} was monitored by a flowmeter (Cole-Parmer). A peek tube guided the airflow to the fly's antennae. For mixtures, the headspaces of the two odors (0.5 l min^{-1} each) were passing through "a mixing peek chamber" to mix the two headspaces before it was delivered through a common Teflon tube (1 mm diameter) to the fly's antennae. In case of single odor, the airflow was compensated by replacing the other odor with clean air (0.5 l min^{-1}). Odors were applied

during frames 8–15 (i.e. after 2 s from the start of recording for 2 s). 1.5-2 mins of clean air were applied between odors, in order to flush any residues of odors and to let the neurons go back to its resting phase.

The flow of the individual odors and the mixtures were monitored by the photo ionization detector (Aurora Scientific) which was placed at the opening of the odor delivery tube. To check whether there is interaction between the two headspaces of the two odors when they are mixed, we performed SPME GC-MS. SPME fiber was placed in the nozzle of the odor delivery system. 10 times of 2 sec odor were applied on the SPME fiber with 3 sec intervals immediately before injected into the GC-MS.

Analysis of calcium signals.

Data were analyzed with custom written IDL software (ITT Visual Information Solutions) provided by Mathias Ditzen as previously described (Stökl et al., 2010; Strutz et al., 2012). Each imaging plane was analyzed separately. All recordings were manually corrected for movement. The raw fluorescence signals were converted to $\Delta F/F_0$, where F_0 is the averaged baseline fluorescence values of 2 seconds before the odor onset (i.e. 0 to 7 frames). For the average $\Delta F/F_0$, average of frames 11 to 18 was calculated for each trail and averaged among trails. All images were compared with a published *in vivo* 3D atlas of the antennal lobe (Grabe et al., 2015). The glomeruli could be reliably identified from the baseline of GCaMP6s, GCaMP6f or GCaMP3.0.

To access the strength of the inhibition, we calculated the “peak responses difference” by subtracting the $\Delta F/F_0$ of the mixture from the single odor, and then data was normalized to the maximum value within a glomerulus.

T-maze experiments.

T-maze experiments were carried out as shown in (Figure 8G). Flies of different genotypes were starved for 24h before they were tested separately under identical

conditions. The preference index was calculates as (O-C)/T, where O is the number of flies in the test arm (i.e. odor arm in Figure 8G, and mixture arm in Figure 8H), C is the number of flies in the control arm (i.e. mineral oil arm in Figure 8G, and individual odor arm in Figure 8H), and T is the total number of flies used in each trail (20 flies). Each trail lasted for 20 minutes.

Expression of receptors (RT-PCR)

Pan-neuronal driver, neuronal synaptobrevin (nsyb-Gal4) was crossed with UAS-dicer2 and the corresponding UAS-RNAi. Flies were raised at 18^{o}C. 2 days old flies were heat shocked at 30^{0}C for 3 days to relive Gal80 repression before they were moved to 25^{0}C prior dissection (Figure S4A, right panel). 50-70 female brains were dissected and total RNA was extracted using Tizol (Sigma). 2 µg from each RNA were used to generate the cDNA. RT-PCR was performed using SuperScript One-Step RT-PCR (invitrogen) according to the manufacturer's instructions. Primers for RT-PCR as follow: Rdl-F: GCGTATAGAAAACGACCTGGTG; Rdl-R: GGACACGATGCGGTTATAGTCA; GABABR2-F: GTAAAGCTCGCCTTGGGTCA; GABABR2-R: CTGGCCTTGGCTATGGGATC; GluClα-F: CCTACCTCGCTTCACACTGG; GluClα-R: CCGGTACTGCTCCTTGATCC; Rp49-F: CCAAGATCGTGAAGAAGCGC; Rp49-R: CTTCTTGAATCCGGTGGGCA.

Immunostaining and microscopy

Whole-mount immunofluorescence staining was performed as previously described (Vosshall et al. 2000). In short, brains were dissected in 4^{o}C phosphate-buffered saline (PBS) (Ca^{+2}, Mg^{+2} free), fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at 25^{o}C, washed 3-4 times for 1.5-2 hours in total in PBS-T (PBS + 0.3% Triton X-100) and blocked for 1 hour in PBS-T + 5% normal goat serum (NGS) at 25^{o}C before incubation in primary antibody diluted in PBS-T + 5% NGS for 48 hours at 4^{o}C. Brains were washed 3-4 times in PBS-T at 25^{o}C before incubation in secondary antibody for 24

hours at 4°C. After secondary antibodies, brains were washed for 3-4 times for 1.5-2 hours at 25°C in PBS-T before mounted in VectaShield (Vector Labs) on glass slides with bridging coverslips. Stained brains were acquired with Zeiss LSM 880 with a 40X water immersion objective lens. The following primary antibodies were applied: chicken anti-GFP (1:500, Thermo Fisher Scientific), rabbit anti-GABA (1:500, Sigma),and mouse mAb anti-bruchpilot (nc82, 1:30, Developmental Studies Hybridoma Bank [DSHB]); secondary antibodies are Alexa Fluor 488 goat anti-chicken (1:300, Thermo Fisher Scientific), Alexa Fluor 568 goat anti-rabbit (1:300, Thermo Fisher Scientific), Alexa Fluor 633 goat anti-mouse (1:300, Thermo Fisher Scientific).

Statistics

Statistics were computed using either the statistics toolbox in R for the FlyWalk data (https://www.r-project.org/). The rest of the data was statistically computed using GraphPad Prism 7 (https://www.graphpad.com/scientific-software/prism/).

Figures

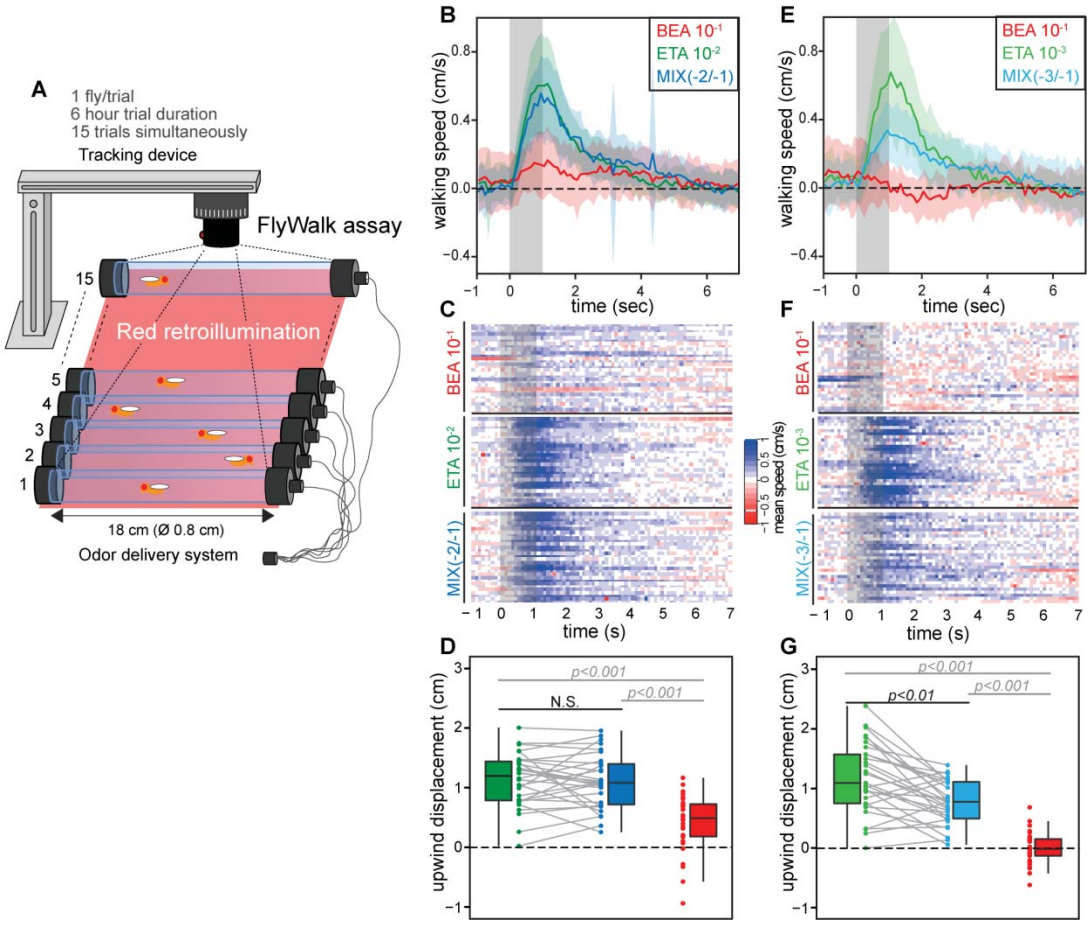

Figure 1. Behavioral responses to binary mixtures of attractive and aversive odors.

(**A**) Schematic diagram of the behavioral paradigm 'Flywalk'. Individual adult females are placed in small glass tubes where odor pulses of single odors or binary mixtures are presented in a continuous airflow background. Adapted from (Stensmyr et al., 2012). See Supplemental Experimental procedures and (Steck et al., 2012; Thoma et al., 2014) for details.

(**B-D**) Behavioral responses to the high concentration of the attractive odor ethyl acetate (ETA 10^{-2}, dark green), the aversive odor benzaldehyde (BEA 10^{-1}, red), and their binary mixture (MIX (+), dark blue), (**B**) Quantitative responses of individual flies to repeated pulses of ETA, BEA or MIX. Lines represent mean responses of 30 individual fly; shadow represents standard deviation. (**C**) Heat map of the mean upwind speed

trajectories in response to BEA, ETA, and MIX (same data set in (B)). Blue represents increase in upwind speed of a single fly, red means downwind. Each row represents the mean trajectory of a single fly. Gray bars in (B and C) represent the odor pulse (1 sec). (**D**) Box plots represent net upwind displacement (data set of B and C) of 4 sec from pulse onset. No significant differences between the flies' responses to the attractive odor (ETA 10^{-2}) and (MIX (+)). Dots and gray lines represent individual trails (Wilcoxon signed rank test).

(**E-G**) Same as (B-D) but for the lower concentration of the attractive odor (ETA, 10^{-3}). (**E**) Mean responses of 30 adult females to ETA 10^{-3} (green), BEA 10^{-1} (red), and MIX (-) (cyan). The attractiveness to MIX (-) is suppressed compared to the response to the attractive odor alone (ETA 10^{-3}). (**F**) Color-coded mean response trajectories for individual flies in each raw. (**G**) Box-plots (data set of E and F) show that MIX (-) is less attractive than ETA (10^{-3}) (Wilcoxon signed rank test). Black lines in the box plots represent the medians.

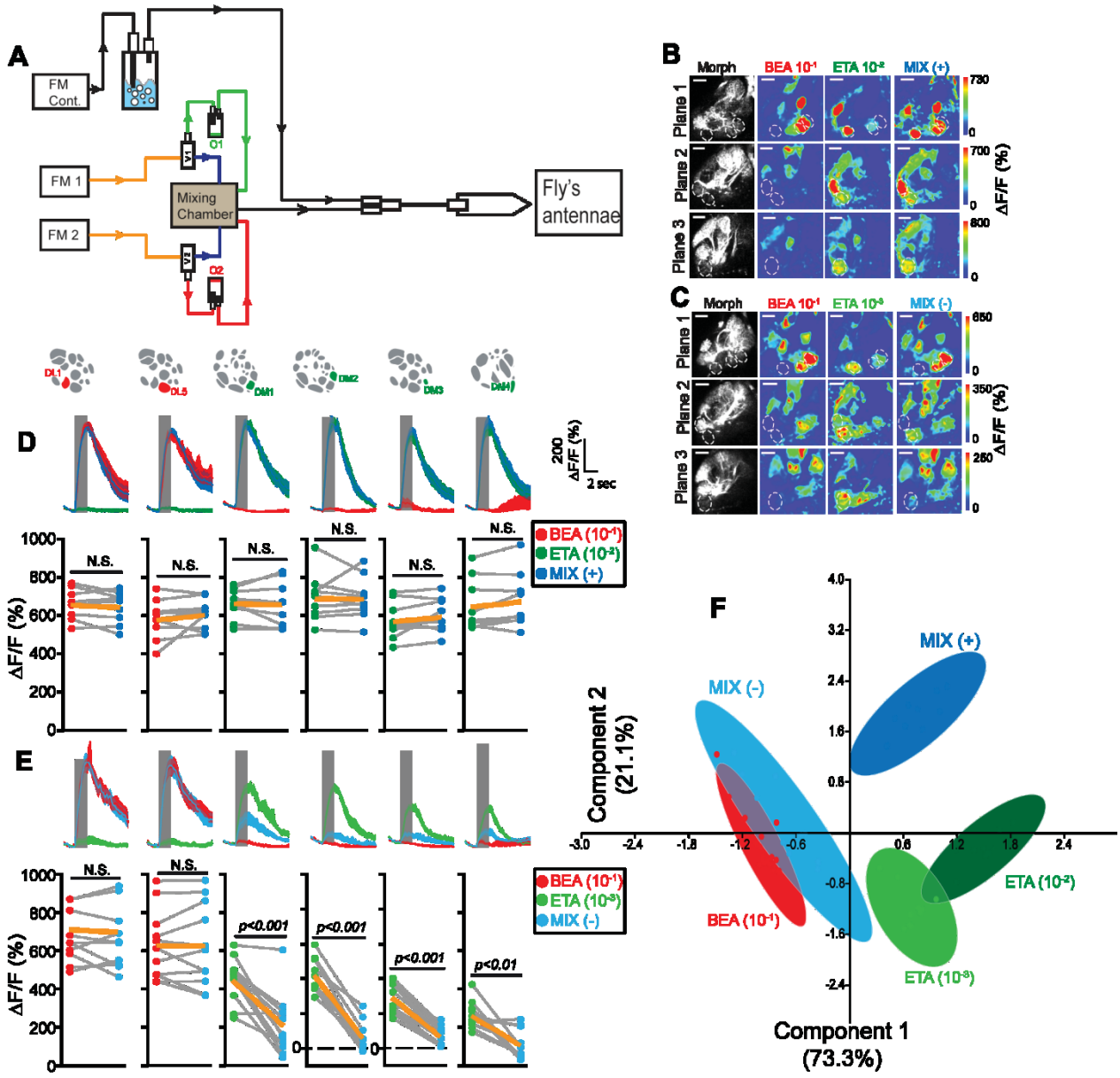

Figure 2. Mixture interactions at the PN level.

(**A**) Schematic drawing of the olfactory delivery system connected with the 2-photon microscope for applying mixtures. See Supplemental Experimental procedures.

(**B, C**) Examples of three imaging planes of two-photon imaging of odor-evoked calcium signals in PNs. GCaMP6s was expressed in PNs under control of the *GH146-Gal4* line. (**B**) Responses to odor stimulation. Gray-scale images represent the antennal lobe structure of three imaging planes (with identified glomeruli highlighted in D).

Pseudocolored images show the responses in those three planes to 2 s odor stimulation of the high concentration of ETA (10^{-2}), BEA 10^{-1}, or their (MIX (+)). Dotted circles mark glomeruli DL1, DL5, DM1, DM2, DM3, and DM4. (**C**) Same as (B) but for the responses of the low concentration of ETA (10^{-3}), BEA 10^{-1}, and their (MIX (-)) (with identified glomeruli highlighted in E).

(**D**) Mean PN activity of strongest activated *aversive* and *attractive* glomeruli in response to stimulation with ETA 10^{-2} (dark green), BEA 10^{-1} (Red) and their (MIX (+)) (dark blue). Upper panel, averaged traces of calcium signals over times (lines), shadows represent SEM; gray bar represents 2 s odor stimulation. Lower panel, mean fluorescence signals within 2 s after odor onset (for data analysis see Supplemental Experimental procedures). Color codes are the same as the upper panel. Gray lines represent individual flies, orange lines show the mean (n=9, paired t-test). Color code of glomeruli reflects activation by aversive (red) or attractive (green) odors.

(**E**) Same as in (D) for the low concentration of ETA (10^{-3}) (green), BEA 10^{-1} (red), and their (MIX (-)) (cyan). 1 replicate is out of the range in glomerulus DL1 (n=11, paired t-test).

(**F**) Principle component analysis (PCA) of the six most activated glomeruli during stimulation with attractive, aversive odors and their binary mixtures (same glomeruli as in D and E). Dots represent individual trails. Two data sets (from E and F, see below) are combined for BEA 10^{-1}. Shadows represent 95% ellipses for each odor. No significant difference between (MIX (-)) and BEA 10^{-1} (One-way ANOSIM, Rho similarity index), indicating that the response to the low concentration mixture (MIX (-)) is indistinguishable from BEA 10^{-1} due to the inhibition of the *attractive* glomeruli (E).

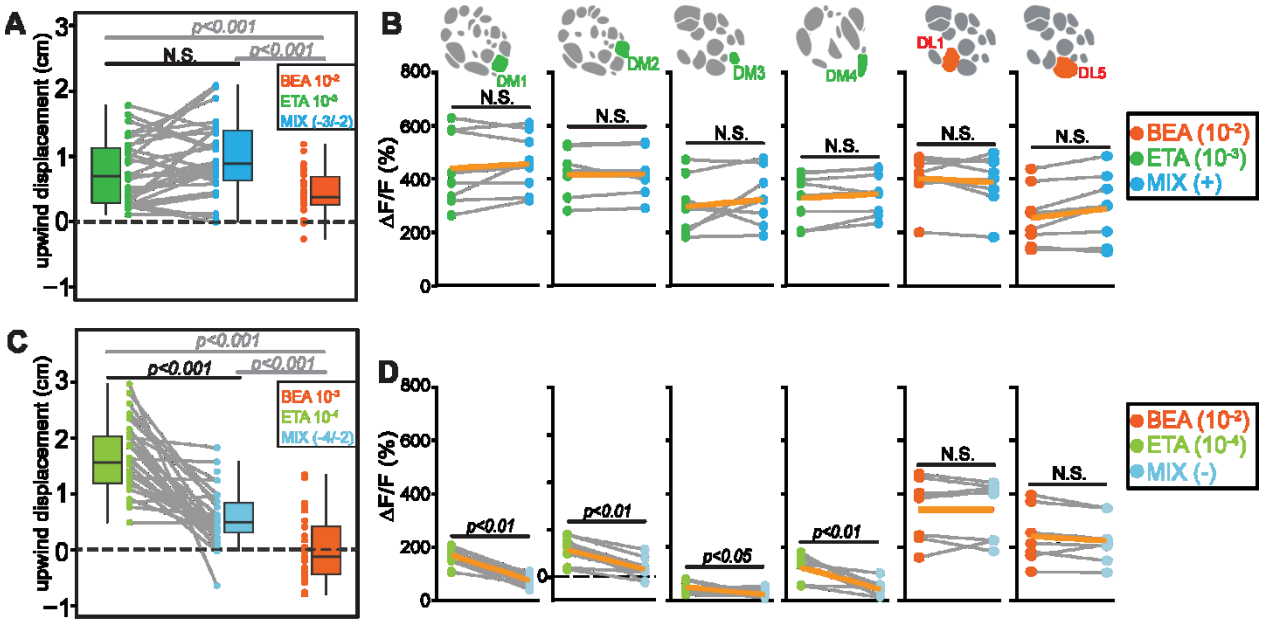

Figure 3. Mixture interactions are ratio-dependent.

(**A, C**) Behavioral responses from the 'Flywalk' during ETA and BEA stimulation of different concentrations and their binary mixtures. (**A**) Net upwind displacement of wild type flies within 4 s from encountering pulses of ETA 10^{-3} (green), BEA 10^{-2} (light red), and MIX (+)(cyan). Flies were attracted to (MIX (+)) in the same manner as to ETA (10^{-3}). (**C**) Statistical analysis of the behavioral responses of the lower concentration of ETA (10^{-4}, light green), BEA 10^{-2} (light red), and MIX (-) (light cyan). Dots and gray lines represent individual trials (n=30, Wilcoxon signed rank test). Black lines in the box plots represent the medians.

(**B, D**) Two-photon calcium imaging of PNs expressing GCaMP6s. Fluorescence signals after stimulation with the same odor concentrations as in (A) or (C), respectively. Individual trails are shown as dots and gray lines, averaged responses are given as orange lines (n=8 for each separate experiment, paired t-test).

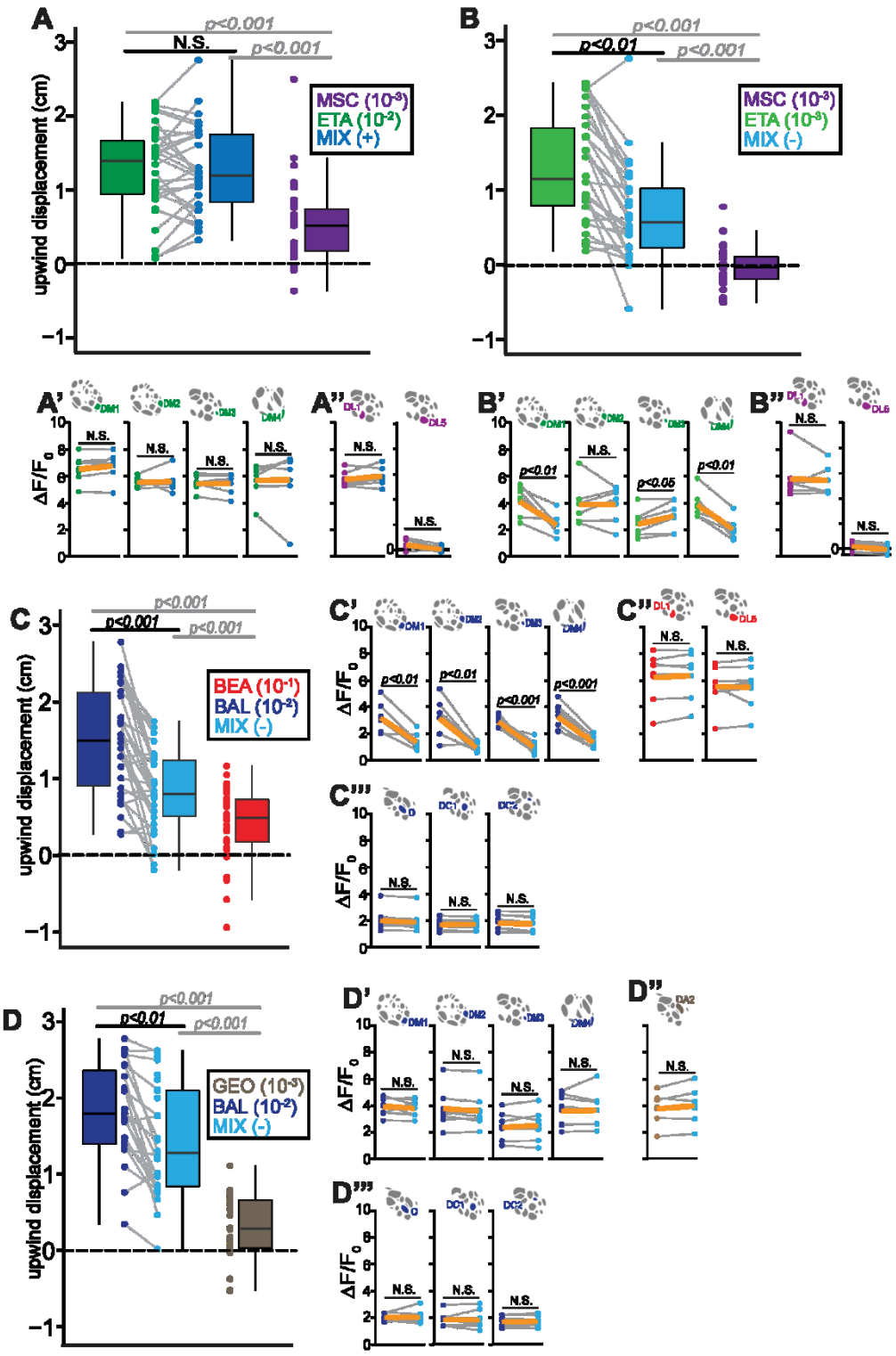

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Figure 4. Different binary mixtures reveal glomerulus-specific mixture inhibitions.

(**A, B, C, D**) Behavioral responses from the 'Flywalk' to different attractive and aversive odors and their binary mixtures. (A-B) Stimulation with methyl salicylate (MSC, 10^{-3}), and ETA (10^{-2}), and their binary mixture (MIX (+)) (A) and to the same concentration of MSC, low concentration ETA (10^{-3}) and MIX (-) (B). Stimulation with BEA (10^{-1}), balsamic vinegar (BAL, 10^{-2}) and their binary mixture (MIX (-)) (C). Stimulation with geosmin (GEO, 10^{-3}), BAL (10^{-2}) and the mixture (MIX (-)) (D). Dots and gray lines represent individual trails. Boxes represent the median (black line) and the first and third quartiles. n=30 for each experiment, Wilcoxon signed rank test.

(**A', A''**) GCaMP signals after stimulation with MSC, ETA and the mixture of PNs of the *attractive* glomeruli (A) and *aversive* glomeruli (A'). MSC does not activate DL5 (n=6, paired t-test).

(**B', B''**) Same as in (A, A') for the lower concentration of the attractive odor (ETA, 10^{-3}) (n=6, paired t-test).

(**C'-C'''**) Two-photon calcium imaging of PNs expressing GCaMP6s. Fluorescence signals after stimulation with the same odor concentrations as in (C) from PNs of *attractive* glomeruli (C' and C''') and *aversive* glomeruli (C'') (n=6, paired t-test).

(**D'-D'''**) Fluorescence signals after stimulation with the same odor concentrations as in (D) from PNs of attractive glomeruli (D' and D''') and aversive glomeruli (D'') (n=6, paired t-test) Dots and gray bars represent individual trails. Mean is shown as orange line.

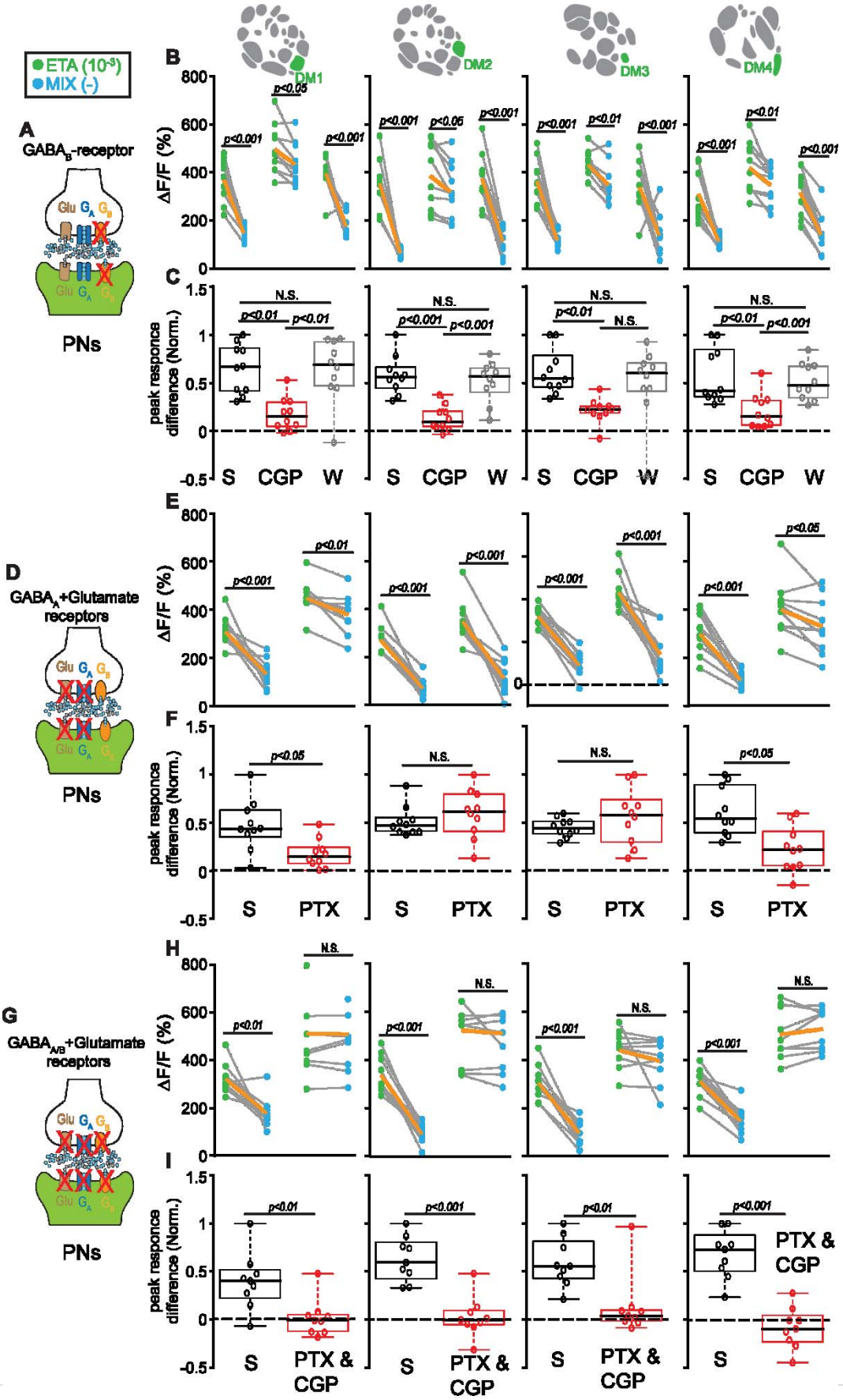

ETA (10-3)
MIX (-)
A
GABAB-receptor
PNs
B
DM1
DM2
DM3
DM4
ΔF/F (%)
C
peak response difference (Norm.)
S
CGP
W
D
GABAA+Glutamate receptors
PNs
E
F
PTX
G
GABAA/B+Glutamate receptors
PNs
H
I
PTX & CGP
N.S.

Figure 5. Inhibitory mixture interactions can be blocked by GABA- and glutamate antagonists.

(**A**) Schematic drawing of the experiment: $GABA_B$ antagonist CGP54626 (50 µM) is applied while calcium responses of PNs are monitored (green).

(**B**) Calcium signals from the four *attractive* glomeruli (DM1, DM2, DM3, and DM4) showing the effect of CGP54626 (CGP) compared to saline (S) or wash-out (W) during stimulation with ETA 10^{-3} (green) and MIX (-) (cyan). The inhibition to the mixture is significantly and reversibly suppressed by blocking $GABA_B$ receptors in all four glomeruli. Dots and gray lines represent single animals, mean is given by orange lines (n=10, paired t-test).

(**C**) Box plots representing normalized peak response differences of calcium signals from the four *attractive* glomeruli shown in (B). Differences were calculated by subtracting fluorescence signals to the mixture from those to ETA during the different treatments (i.e. 1 represents strongest mixture inhibition, while 0 means no inhibition). Black lines represent median, circles show individual animals. The inhibition in the four *attractive* glomeruli is significantly suppressed during application of the $GABA_B$ receptor antagonist (one-way ANOVA with Bonferroni's multiple comparisons test).

(**D**) Schematic drawing of the experiment: $GABA_A$ and glutamate receptor antagonist picrotoxin (100 µM) is applied while calcium responses of PNs are monitored (green).

(**E**) Calcium signals from the four *attractive* glomeruli showing the effect of picrotoxin on the inhibition upon stimulation with MIX (-) (n=10, paired t-test) compared to saline. Picrotoxin could not be washed-out as described in previous studies.

(**F**) Box plots representing normalized peak response differences of calcium signals from the four *attractive* glomeruli shown in (E). The mixture inhibition is abolished in two glomeruli (DM1 and DM4) (n=10, student's t-test).

(**G**) Schematic drawing of the experiment: a mixture of picrotoxin (100 µM) and CGP54626 (50 µM) was applied to block $GABA_A$, $GABA_B$ and glutamate receptors while calcium responses of PNs are monitored (green).

(**H**) Calcium signals from the four *attractive* glomeruli showing the effect of picrotoxin combined with CGP54626 on the inhibition upon stimulation with MIX (-) (n=9, paired t-test) compared to saline. In DM1, one data point in ETA and other in MIX are out of range of the graph. One trail is out of range of the graph in DM2.

(**I**) Box plots representing normalized peak response differences of calcium signals from the four *attractive* glomeruli shown in (H). The mixture inhibition is completely abolished in all four glomeruli compared to saline (n=9, student's t-test).

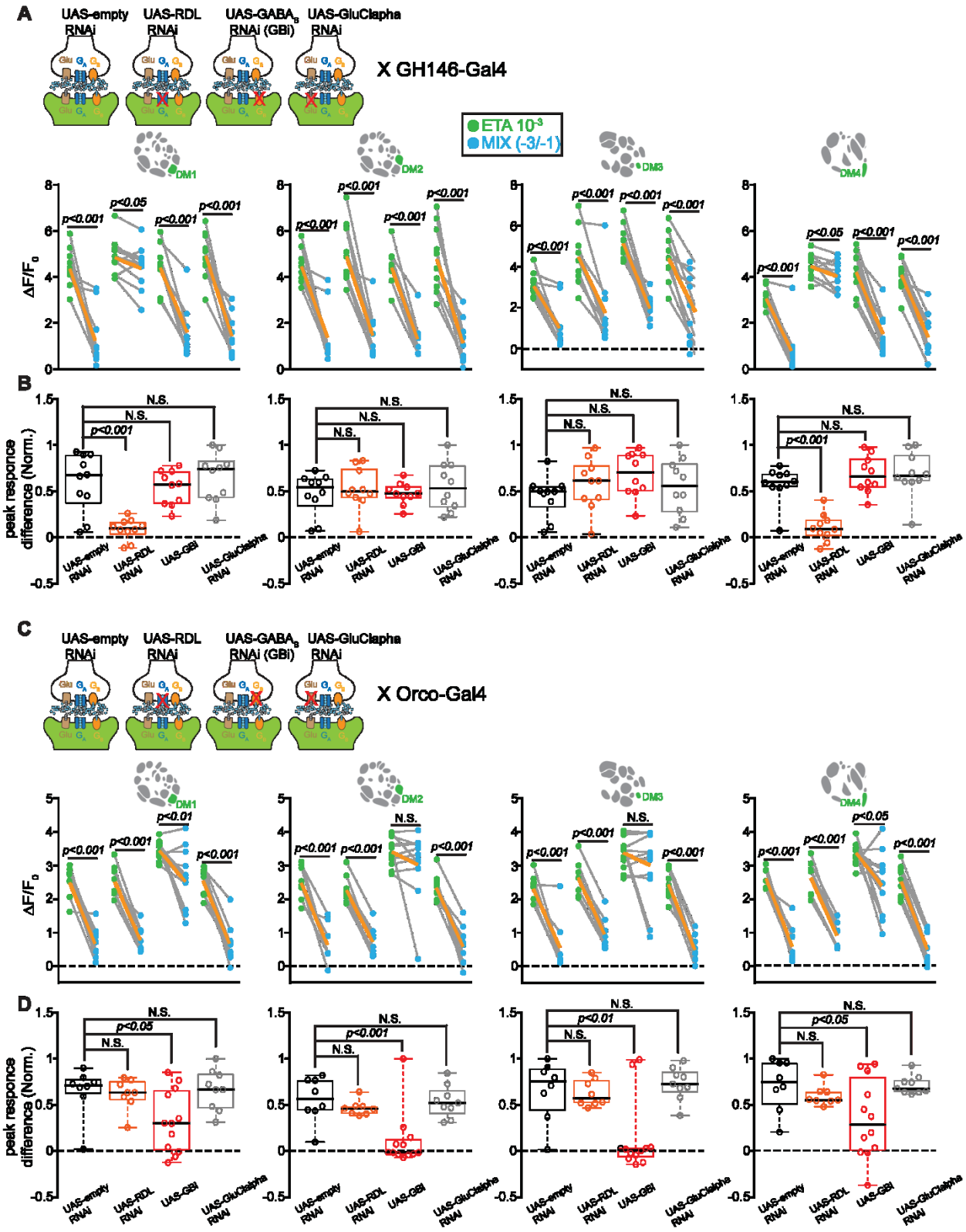

A
UAS-empty RNAi
UAS-RDL RNAi
UAS-GABA$_B$ RNAi (GBi)
UAS-GluClapha RNAi
X GH146-Gal4
ETA 10^{-3}
MIX (-3/-1)
DM1
DM2
DM3
DM4
ΔF/F$_0$
B
peak responce difference (Norm.)
N.S.
p<0.001
p<0.05
p<0.01
UAS-empty RNAi
UAS-RDL RNAi
UAS-GBi
UAS-GluClapha RNAi
C
X Orco-Gal4
D

Figure 6. Pre- and postsynaptic inhibitions are responsible for mixture interaction.

(**A**) Above, schematic drawing of the experiment: blocking either $GABA_A$, $GABA_B$ or glutamate receptors selectively in PNs using specific RNAi-lines (*UAS-Rdl RNAi*, *UAS-GBi* and *UAS-Gluclalpha RNAi*), while calcium responses of PNs are monitored (green). Empty-RNAi serves as the positive control. Below, calcium signals of PNs of the four *attractive* glomeruli (DM1, DM2, DM3, and DM4) during stimulation with ETA (10^{-3}; green dots) and MIX (-) (cyan dots) (n=10, paired t-test). Individual animals are shown by grey lines; mean is given by orange lines.

(**B**) Box plots representing normalized peak response differences of calcium signals from the four *attractive* glomeruli shown in (A). Black lines represent median, circles show individual animals. Silencing $GABA_A$ receptors in PNs suppresses significantly the mixture inhibition in two glomeruli (DM1 and DM4). Silencing either $GABA_B$ or glutamate receptors does not have any effect on the inhibition (one way ANOVA with Bonferroni's multiple comparisons test).

(**C**) Above, schematic drawing of the experiment: same RNAi lines as in (A) are selectively expressed in OSNs, while calcium responses of PNs are monitored (green). Empty-RNAi serves again as the positive control. Below, calcium signals of PNs of the four *attractive* glomeruli during stimulation with ETA (10^{-3}; green dots) and MIX (-) (cyan dots) (n=8-12, paired t-test).

(**D**) Box plots representing normalized peak response differences of calcium signals from the four *attractive* glomeruli shown in (C). Silencing $GABA_B$ receptors in OSNs completely abolished the mixture inhibition in two glomeruli (DM2 and DM3) and significantly reduces the inhibition in the other two glomeruli (DM1 and DM4). Silencing either $GABA_A$ or glutamate receptors does not have any effect on the inhibition (one way ANOVA with Bonferroni's multiple comparisons test).

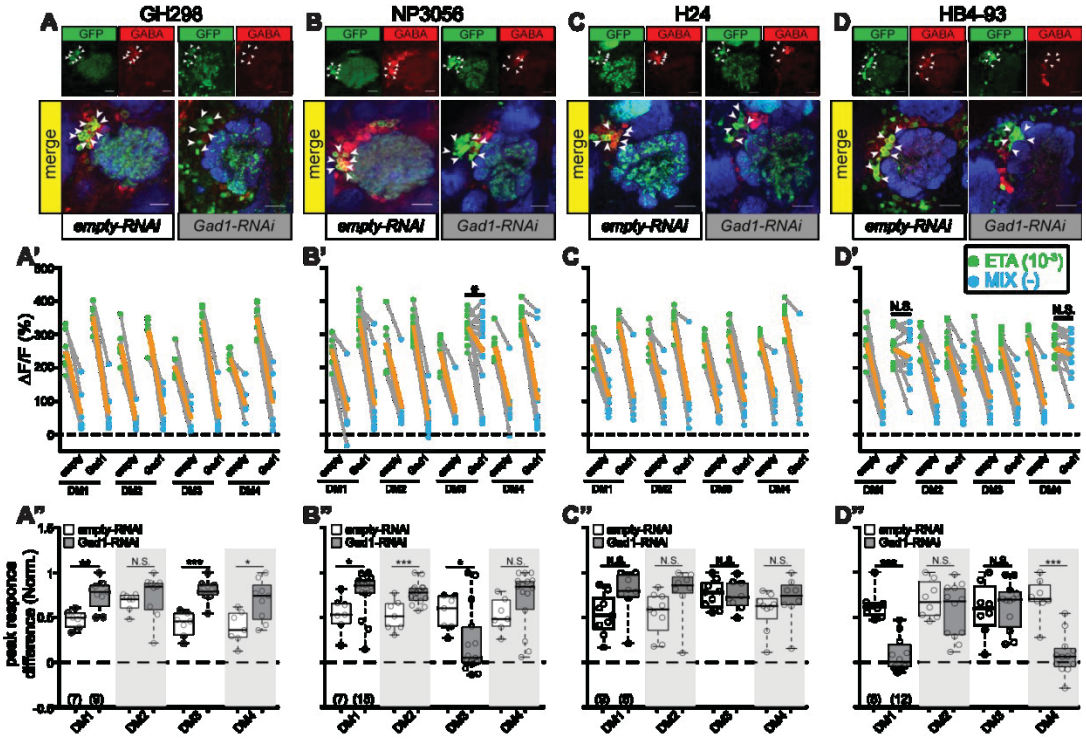


Figure 7 Different subsets of GABAergic LNs are responsible for the mixture inhibition in the *attractive* glomeruli

(**A-D**) Immunostaining against GFP (Green) and GABA (Red) of ALs of different Gal4 lines (from left to right: GH298 Gal4, NP3056 Gal4, H24 Gal4 and HB4-93 Gal4) with either control RNAi or Gad1 RNAi (Silenced GABA production). Arrow heads point to the somata of LNs labelled in different lines. Scale bare=20 µm.

(**E, F, G, H**) GCaMP signals after stimulation with ETA (green) or the mixture (cyan) from PNs of the four *attractive* glomeruli for either intact (empty RNAi) or silenced GABA production (Gad1 RNAi) in GH298 Gal4 (E), NP3056 Gal4 (F), H24 Gal4 (G), and HB4-93 Gal4 (H). Individual trails are represented by dots and gray lines. Orange lines represent mean values. n=7-15 paired t-test between ETA (green dots) and MIX (-) (cyan dots) Paired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

(**E', F', G', H'**) Box plots represent the normalized peak response differences between ETA and the mixture calculated from the GCaMP signal in (E, F, G, H) for GH298 Gal4 (E'), NP3056 Gal4 (F'), H24 Gal4 (G'), and HB4-93 Gal4 (H'). Boxes represent median

and first and third quartiles. empty RNAi are white boxes, Gad1 RNAi are gray boxes. Student t-test (p* < 0.05; **p < 0.01; ***p < 0.001)

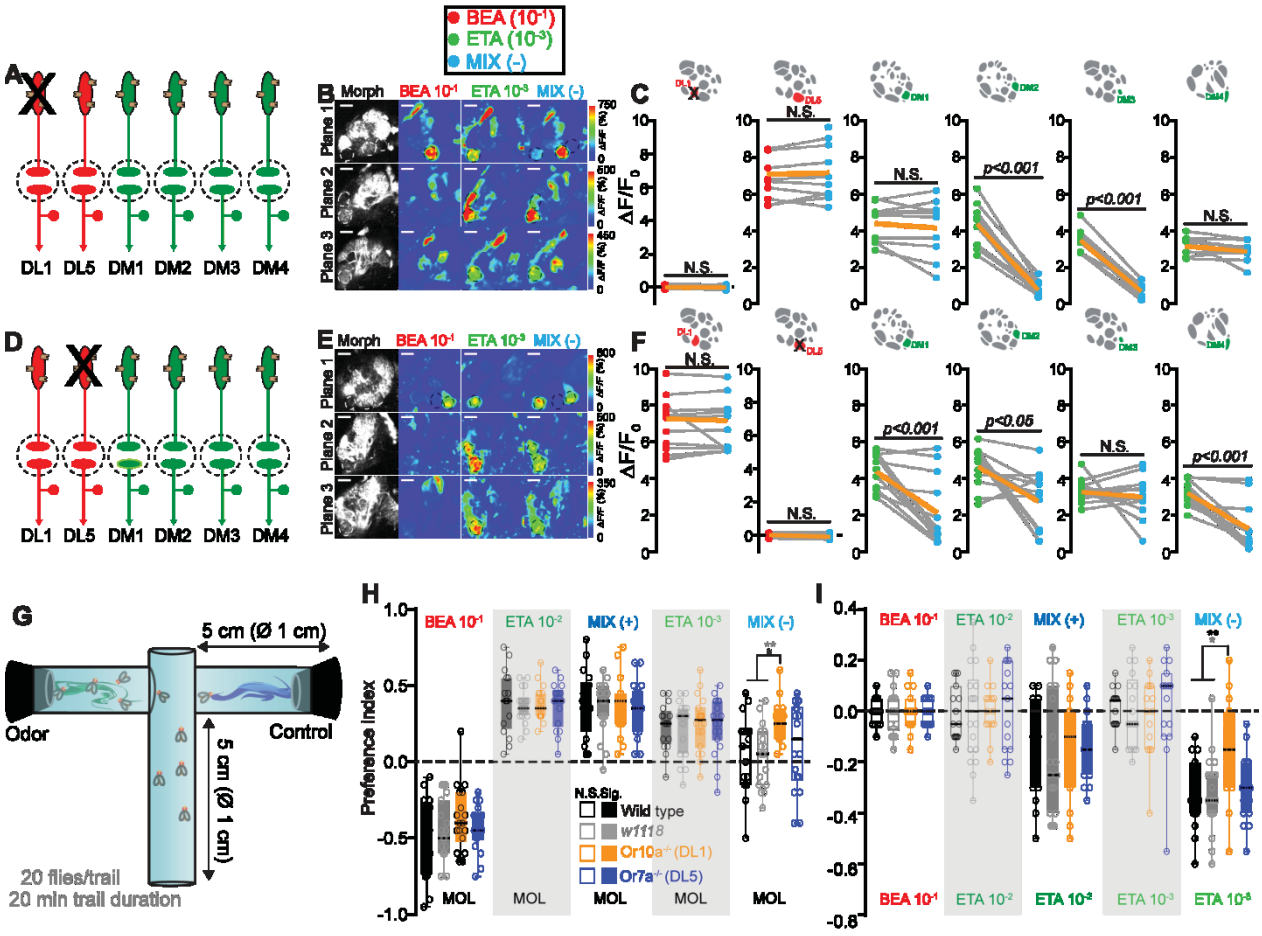


Figure 8. Genetic manipulation of specific ORs reveals glomerulus-specific inhibition.

(**A**) Schematic drawing of the experiment: Or10a expressing OSNs (which target glomerulus DL1) are not functional in a Or10a$^{-/-}$ mutant background. Color code of glomeruli reflects activation by aversive (red) or attractive (green) odors.

(**B**) Gray-scale images show the antennal lobe structure in different focal planes highlighting the *attractive* (DM1, DM2, DM3, and DM4) and *aversive* glomeruli (DL1 and

DL5) with dotted circles. Representative pseudo-colored two photon images of calcium signals from PNs during odor stimulation from the respective focal planes in flies with an Or10a$^{-/-}$ background.

(**C**) Calcium signals of PNs of 6 glomeruli to either BEA (10^{-1}; red dots), ETA (10^{-3}; green dots) or MIX (-) cyan dots) from Or10a$^{-/-}$ flies. Gray lines reveal individual animals, mean is given by orange lines (n=10, paired t-test).

(**D**) Schematic drawing of the experiment: Same as in (B) with flies having an Or7a$^{-/-}$ mutant background (i.e. glomerulus DL5 does not get any input).

(**E**) Gray-scale and pseudocolored images of different imaging planes highlighting the *attractive* and *aversive* glomeruli that were analyzed further.

(**F**) Calcium signals of 6 glomeruli to either BEA (10^{-1}; red dots), ETA (10^{-3}; green dots) or MIX (-) cyan dots) from Or7a$^{-/-}$ flies (n=12, paired t-test).

(**G**) Schematic drawing of the T-maze assay.

(**H**) Box plots showing behavioral attraction indices in the T-maze of Or10a$^{-/-}$ flies (orange), Or7a$^{-/-}$ flies (purple), wildtype flies (black) and w1118 flies (gray) to BEA (10^{-1}), ETA (10^{-2}, 10^{-3}), MIX (+) and MIX (-) against the solvent mineral oil (MOL). Black lines represent medians (n=15-19, Kruskal-Wallis test with Dunn's multiple comparisons test). Filled boxes are significantly different from zero, empty boxes not (student's t-test). While the control strains and Or7a$^{-/-}$ show no attraction to MIX (-), Or10a$^{-/-}$ flies are highly attracted to the same mixture.

(**I**) Behavioral attraction indices in the T-maze to the mixtures against the corresponding concentration of the attractive odor (ETA). Or10a$^{-/-}$ flies show less aversion to MIX (-) compared to the two control strains, while Or7a$^{-/-}$ flies follow the control flies (n= 15-19).

Supplemental Material

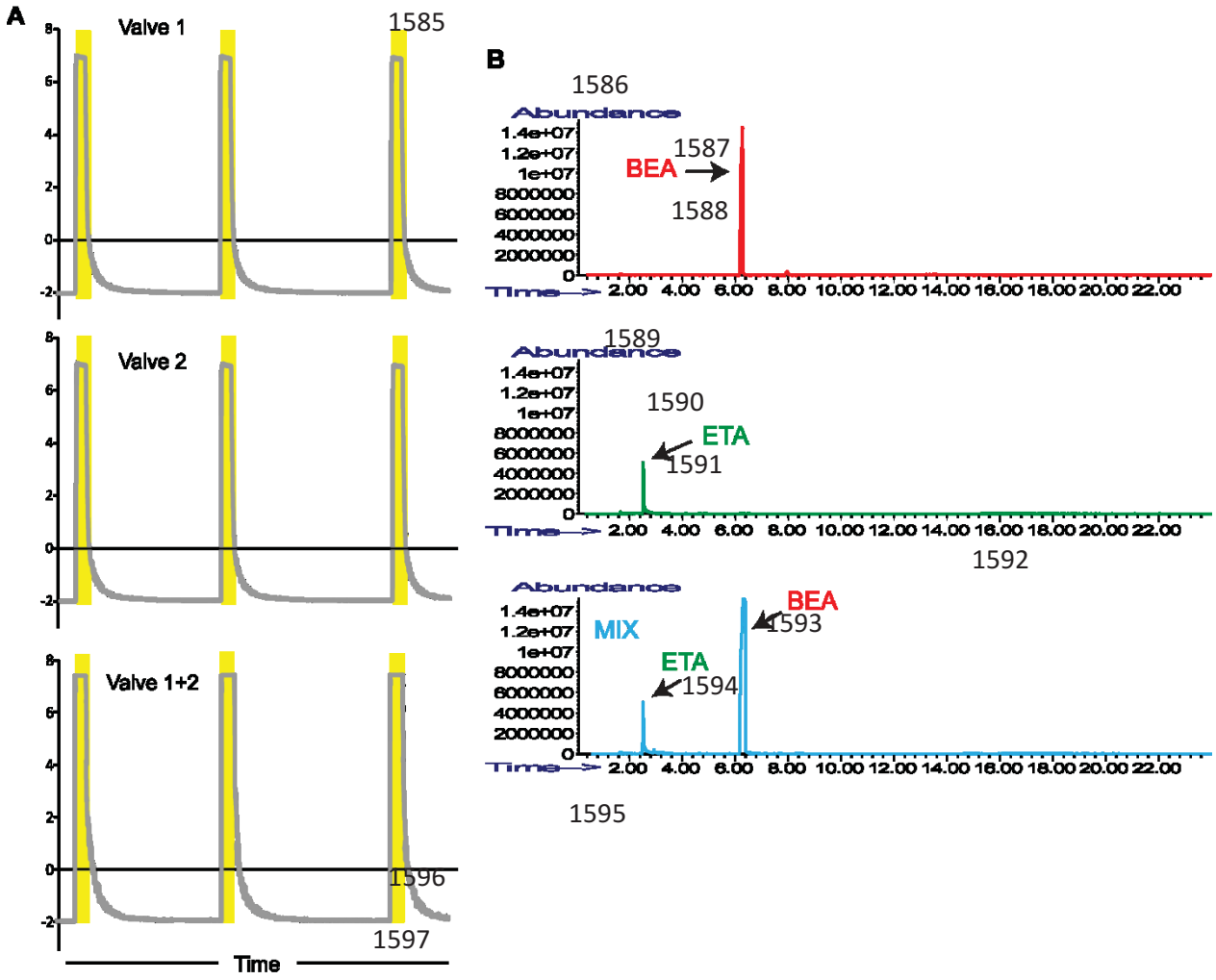


Fig. S1| Checking the odor delivery system

A) PID traces of valve 1 and valve 2 and both together. **B)** GC-MS traces of SPME odor collections of valve 1 and valve 2 and both together.

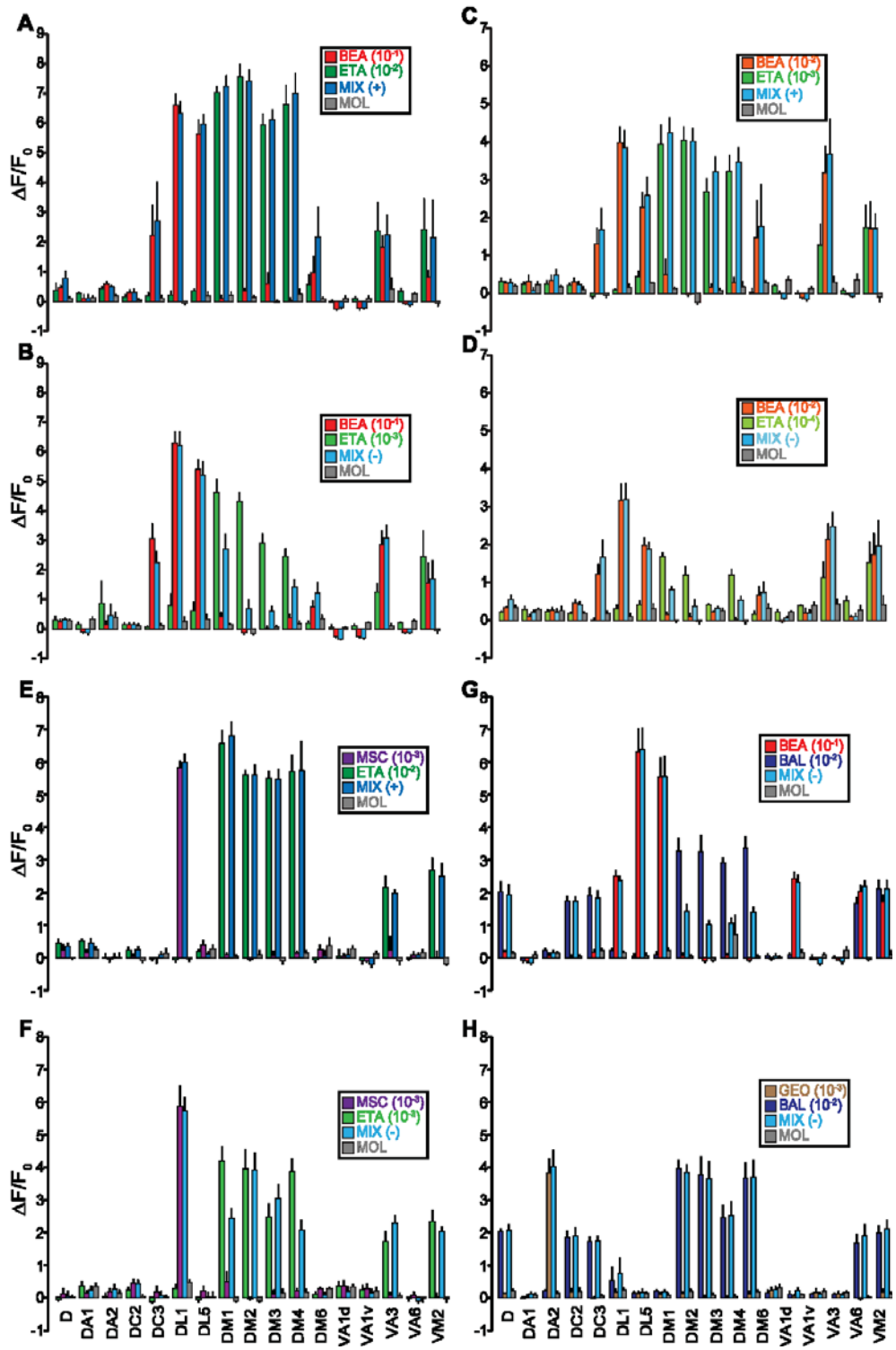
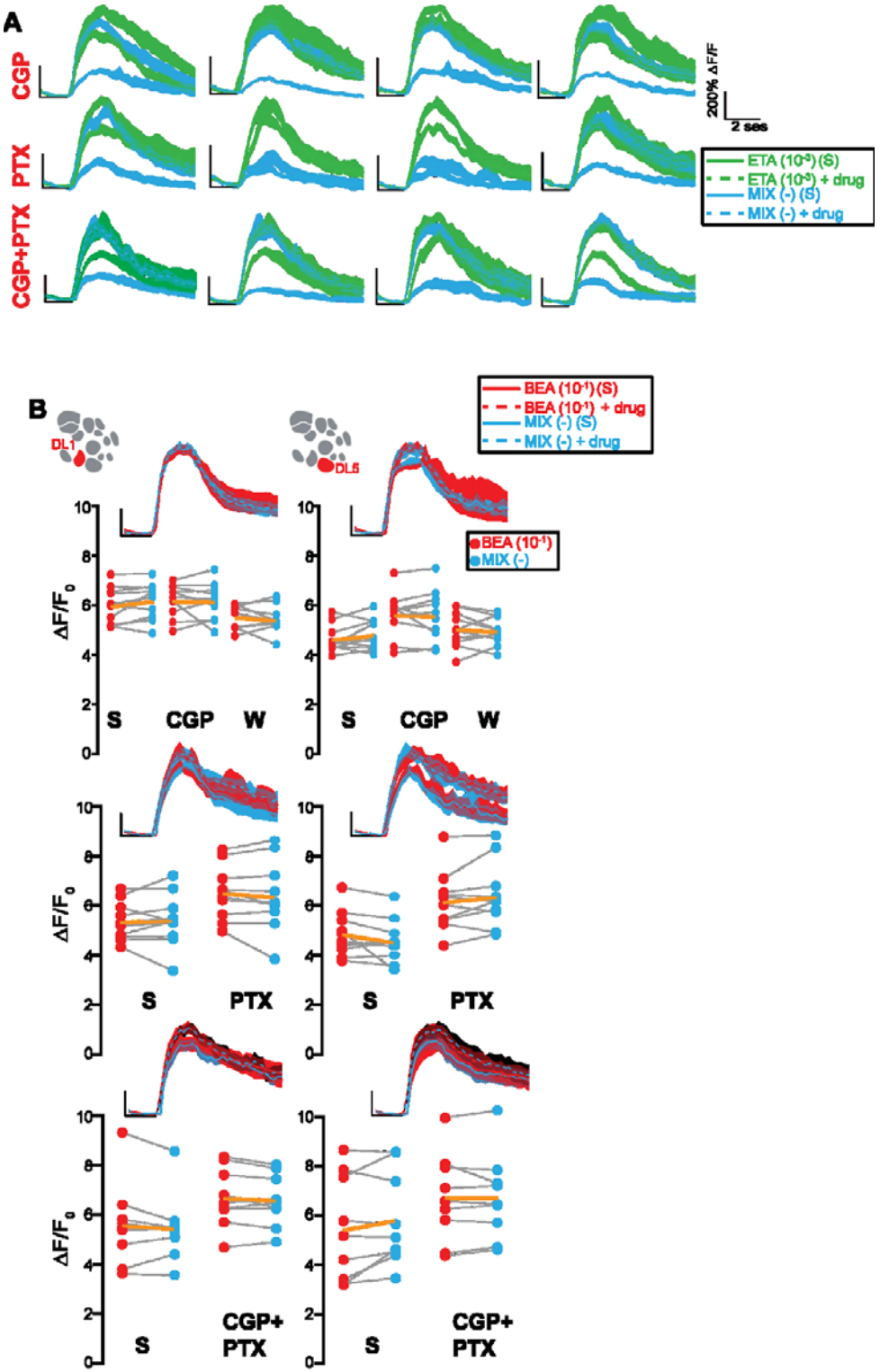


Fig. S2| Glomeruli activation patterns of different binary mixtures

A) Glomeruli activated by ethyl acetate (10^{-2}; green), benzaldehyde (10^{-1}; red) and their mixture (blue). **B)** Glomeruli activated by ethyl acetate (10^{-3}; green), benzaldehyde (10^{-1}; red) and their mixture (blue) **C)** Glomeruli activated by ethyl acetate (10^{-3}; green), benzaldehyde (10^{-2}; red) and their mixture (blue) **D)** Glomeruli activated by ethyl acetate (10^{-4}; green), benzaldehyde (10^{-2}; red) and their mixture (blue) **E)** Glomeruli activated by ethyl acetate (10^{-2}; green), methyl salicylate (10^{-3}; purple) and their mixture (blue) **F)** Glomeruli activated by ethyl acetate (10^{-3}; green), methyl salicylate (10^{-3}; purple) and their mixture (blue) **G)** Glomeruli activated by balsamic vinegar (10^{-2}; dark blue), benzaldehyde (10^{-1}; red) and their mixture (blue) **H)** Glomeruli activated by balsamic vinegar (10^{-2}; dark blue), geosmin (10^{-3}; brown) and their mixture (blue)



1616

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1618 **Fig. S3| Investigating inhibitory mixture interactions using drug application**

A) Calcium-imaging time traces of ethyl acetate and its binary mixture with benzaldehyde with (blue) and without (green) drugs. **B)** Calcium-imaging time traces of benzaldehyde and its binary mixture with ethyl acetate with (blue) and without (red) drugs. As well as the differences in the actual signal.

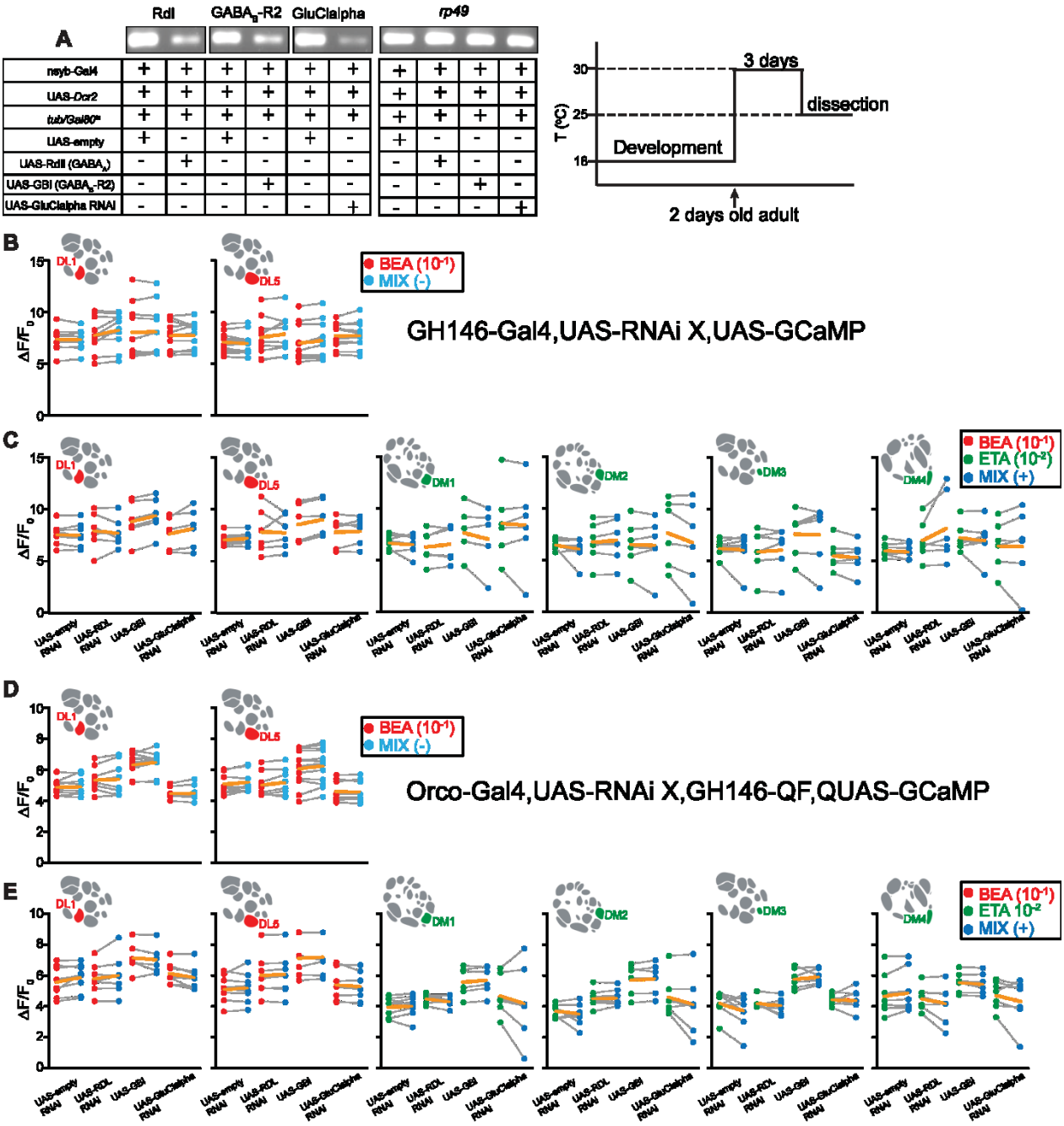


Fig. S4| Calcium-imaging of PNs with RNAi against GABA receptors

A) Left: RT-PCR of the used fly lines. Right: scheme of the experimental protocol **B-E)** Calcium-imaging of single glomeruli (aversive = red; attractive = green).

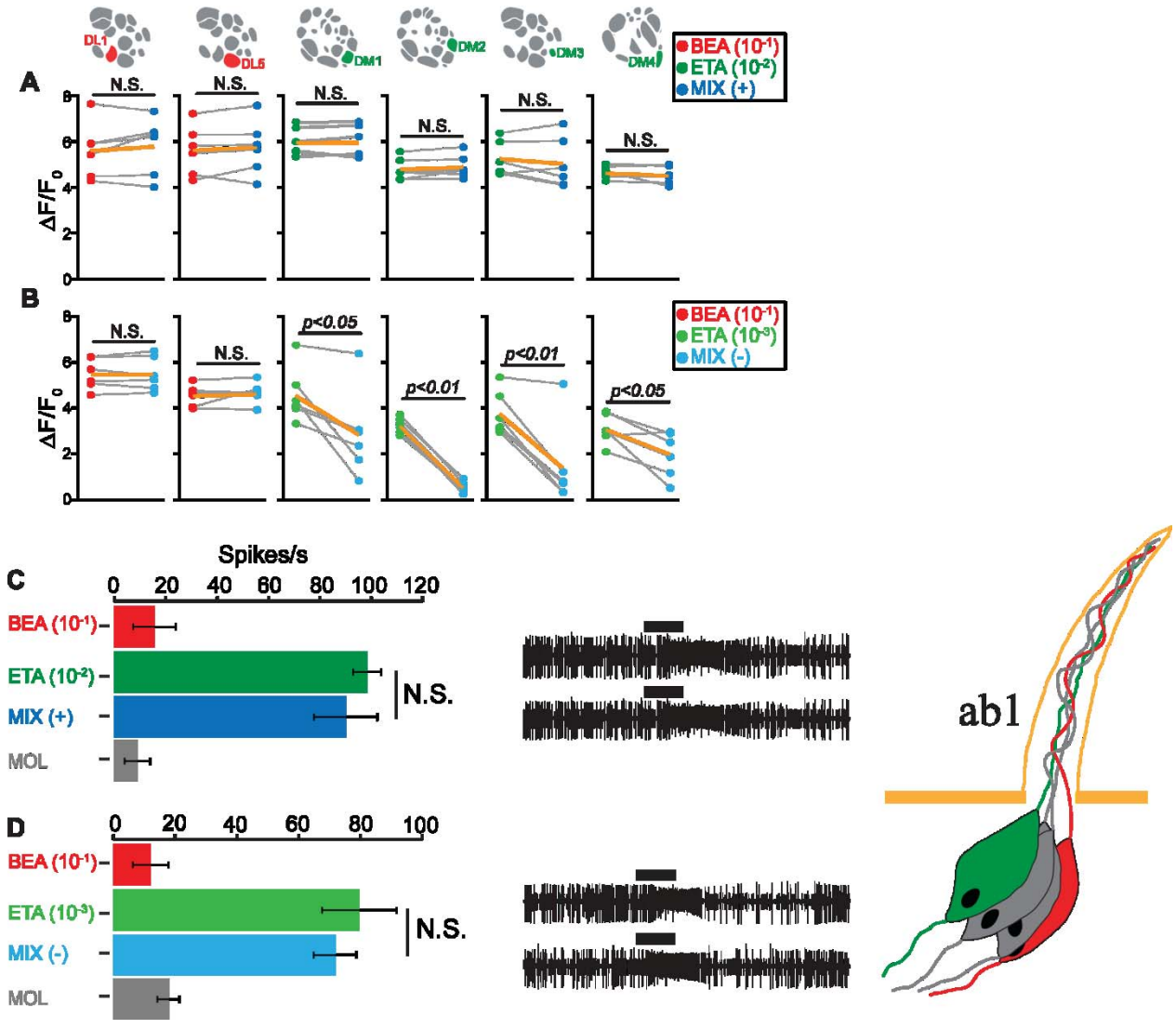


Fig. S5| No inhibition on OSN level

A-B) Calcium-imaging of glomeruli on OSN level. **C-D)** Single sensillum recordings from ab1.

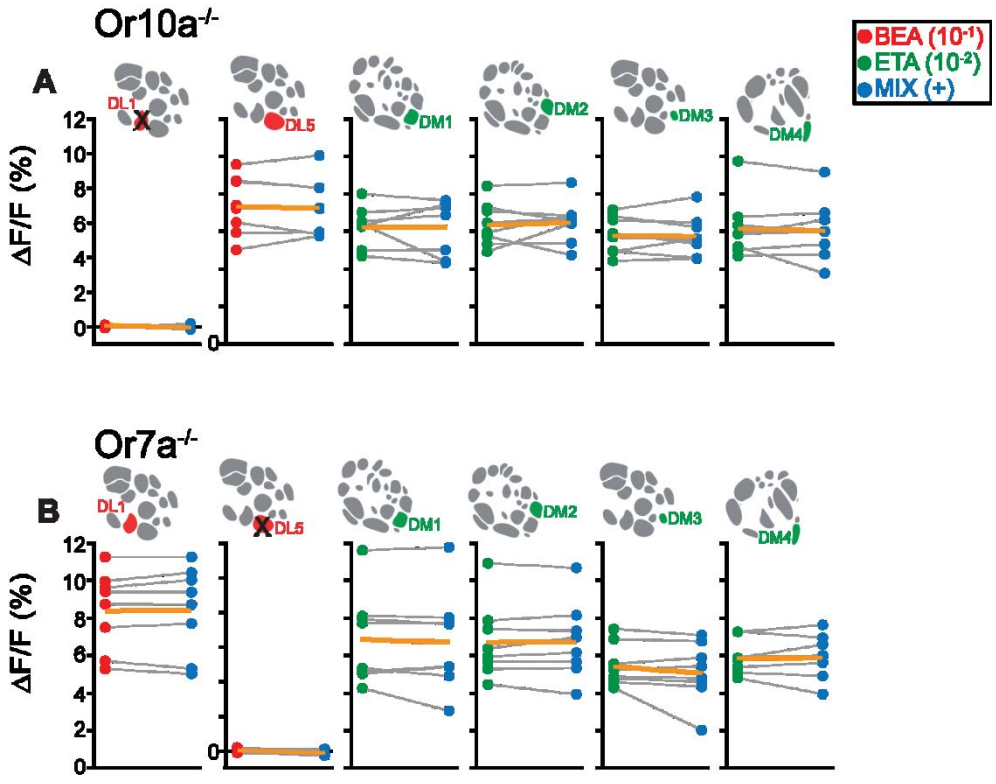


Fig. S6| Calcium-imaging in OR10 mutants and OR7a mutants

A) Calcium-imaging on PN level in OR10 mutants **B)** Calcium-imaging on PN level in OR7a mutants

1643 **Table 1. Flylines**

Figures	Genotypes
Figure 1	Wild-type Canton S
Figure 2	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 3A, 3C	Wild-type Canton S
Figure 3B, 3D	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 4A, 4B, 4C and 4D	Wild-type Canton S
Figure 4A', 4A'', 4B', 4B'', 4C'-4C''' and 4D'-4D'''	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 5	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 6A-6I	UAS-dicer2; GH146-Gal4,UAS-GCaMP6s/UAS-empty RNAi; TM2/TM6B
	UAS-dicer2; GH146-Gal4,UAS-GCaMP6s/UAS-GBi; UAS-GBi/TM6B
	UAS-dicer2; GH146-Gal4,UAS-GCaMP6s/(Cyo); UAS-Rdli/TM6B
	UAS-dicer2; GH146-Gal4,UAS-GCaMP6s/UAS-gluclalpha RNAi; TM2/TM6B
Figure 6J-R	UAS-dicer2; UAS-empty RNAi/Cyo; GH146-QF,QUAS-GCaMP3/Orco-Gal4
	UAS-dicer2; UAS-GBi /(Cyo); GH146-QF,QUAS-GCaMP3/Orco-Gal4
	UAS-dicer2; Orco-Gal4/(Cyo); GH146-QF,QUAS-GCaMP3/UAS-Rdli
	UAS-dicer2; UAS-gluclalpha RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/Orco-Gal4
Figure 7	UAS-dicer2; UAS-empty RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/GH298-Gal4
	UAS-dicer2; UAS-Gad1 RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/GH298-Gal4
	UAS-dicer2; UAS-empty RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/NP3056-Gal4
	UAS-dicer2; UAS-Gad1 RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/NP3056-Gal4
	UAS-dicer2; UAS-empty RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/H24-Gal4
	UAS-dicer2; UAS-Gad1 RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/H24-Gal4

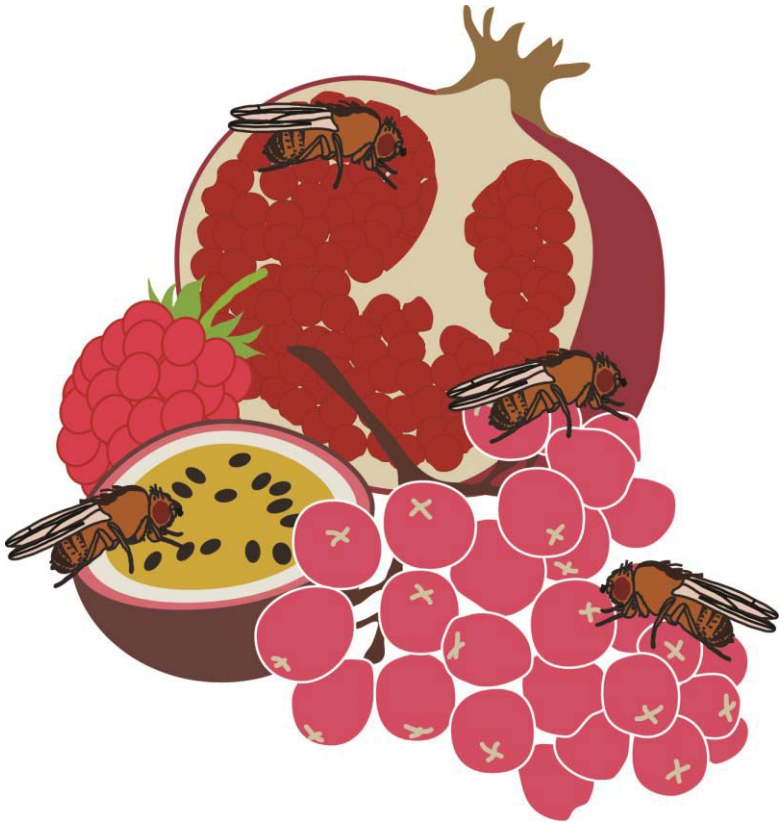
	UAS-dicer2; UAS-empty RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/HB4-93-Gal4
	UAS-dicer2; UAS-Gad1 RNAi/(Cyo); GH146-QF,QUAS-GCaMP3/HB4-93-Gal4
Figure 8A, 8B	$Or10a^{-/-}$; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 8C, 8D	$Or10a^{-/-}$; +/+; +/+
Figure 8E, 8F	$Or7a^{-/-}$; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Figure 8G, 8H	$Or7a^{-/-}$; +/+; +/+
Supp 2	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Supp 3	w-; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Supp 4	Mentioned in the figure
Supp 5	w-; Cyo/Bl; Orco-Gal4/UAS-GCaMP6f
Supp 6A	$Or10a^{-/-}$; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B
Supp 6B	$Or7a^{-/-}$; GH146-Gal4,UAS-GCaMP6s/(Cyo); TM2/TM6B

Manuscript V

Logic Behind Differences in Food Preferences Between Larval and Adult *Drosophila*

Hany K. M. Dweck, Shimaa A. M. Ebrahim, Tom Retzke, Veit Grabe, Jerrit Weißflog, Markus Knaden, Bill S Hansson

Manuscript submitted to Neuron



The Olfactory Logic Behind Food Preference in Larval and Adult *Drosophila*

Hany K. M. Dweck[1, 3, 4, *], Shimaa A. M. Ebrahim[1,3, 4], Tom Retzke[1], Veit Grabe[1], Jerrit Weißflog[2], Ales Svatos[2], Bill S Hansson[1,5], Markus Knaden[1,5,6]

[1]Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, 07745 Jena, Germany

[2]Mass Spectrometry Group, Max Planck Institute for Chemical Ecology, Jena, Germany

[3]Present address: Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520, USA

[4]authors share first authorship

[5]authors share senior authorship

[6]Lead Contact

*Correspondence: hany.dweck@yale.edu, mknaden@ice.mpg.de

SUMMARY

Compared to the substantial work invested in deciphering the molecular and cellular basis of odor coding, our understanding of the relationship between sensory input and behavioral output in larval and adult *Drosophila* is rather weak. Here, we measure behavior generated by larval and adult flies to 34 fruit-headspace extracts and find that larval preference for fruit odors is clearly different from that of adult flies. Next, we provide a functional analysis of the full repertoire of the peripheral olfactory system using the same comprehensive stimulus spectrum. We find that 90 and 53 percent of the olfactory system in larval and adult flies, respectively, are involved in evaluating these fruit odors. Finally, we find that the total amount of olfactory neuronal activity correlates strongly positively with behavioral output in larvae, and correlates weakly negatively in adult flies. These correlations are strongly affected by the activation of Or45a in larvae and Or67a adult flies. Taken together, our results suggest that larval and adult flies have evolved different mechanisms for detection and computation of fruit odors, mechanisms likely mirroring the different life styles of the two developmental stages.

INTRODUCTION

Larval and adult *Drosophila melanogaster* evaluate olfactory information emitted from their ecological niche, decaying fruits (Hansson et al., 2010), via the olfactory system. The larval olfactory system consists of 21 olfactory sensory neurons (OSNs) housed in a single morphological structure, the dorsal organ, located at the tip of the head (Ramaekers et al., 2005). These 21 OSNs express, in addition to the coreceptor Orco, 25 members of the odorant receptor (Or) family. Out of these, 13 are also expressed in the adult (Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2005; Kreher et al., 2008). In contrast, the 48 OSN types of the adult olfactory system are housed in three large antennal basiconic (ab1-ab3), seven small antennal basiconic (ab4-ab10), two antennal intermediate (ai1 and ai2, previously known as at2 and at3, respectively), two antennal trichoid (at1 and at4), four antennal ceoloconic (ac1-ac4) and three palp basiconic (ac1-ac4) sensilla (Couto et al., 2005; Shanbhag et al., 1999). OSNs housed in basiconic, intermediate and trichoid sensilla express 44 Or genes along with the Orco coreceptor (Couto et al., 2005). However, with the exception of Or35a/Orco, OSNs housed in coeloconic sensilla express 12 ionotropic receptor (Ir) genes including three co-receptors, Ir8a, Ir25a and Ir76b (Benton et al., 2009).

There are two ways to address the question of how sensory input is converted into behavioral output. The first is to dissect the circuit of each sensory input from the peripheral OSNs to the primary processing center in the brain, the antennal lobe (AL), and from the AL to higher brain centers like mushroom body and lateral horn, finally leading to a behavioral output. The second is to describe sensory input and behavioral output quantitatively, considering processing of information in the central nervous system as a black box. In the vinegar fly, *Drosophila melanogaster*, several studies have used the second way (Bell and Wilson, 2016; Hernandez-Nunez et al., 2015; Knaden et al., 2012; Kreher et al., 2008; Thoma et al., 2014). However, none of these studies analyzed the sensory input of the entire olfactory system, particularly in adult flies, and none of them dealt with ecologically relevant complex stimuli.

In this study, we measured behavioral responses of larval and adult flies to headspaces of 34 different fruits (Figure 1A). Subsequently, we dissected how these fruit odors are detected by the entire olfactory systems of larval and adult flies. Finally, we correlated the physiological responses with the behavioral output to decipher the logic behind food preference in larval and adult flies.

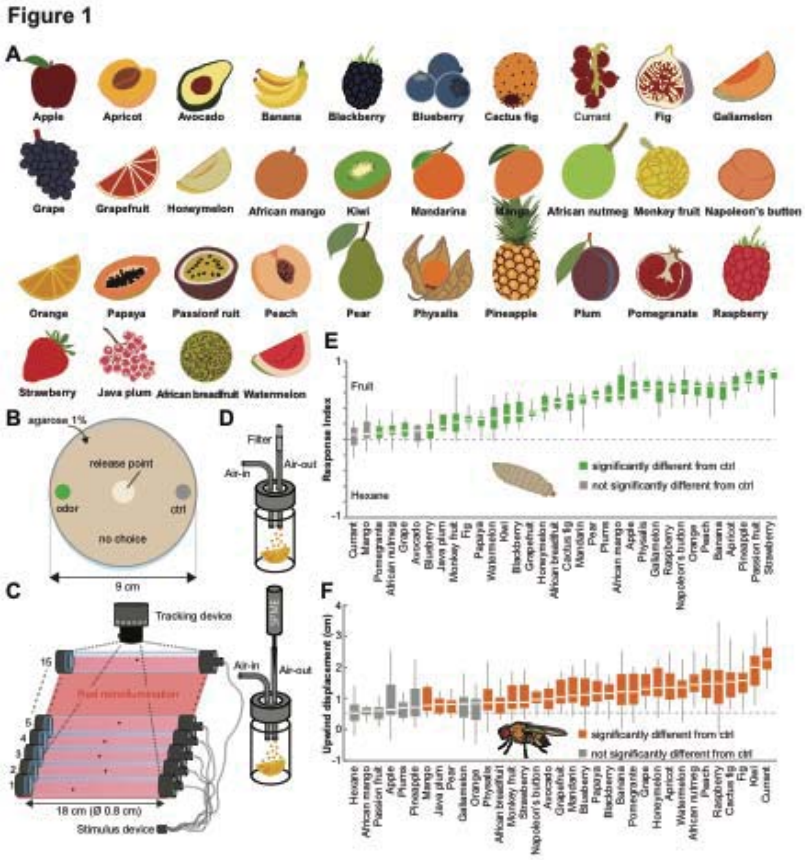


Figure 1. Behavioral responses of larval and adult *Drosophila* to fruit-headspace extracts.

(A) Illustration of 34 different fruits.

(B) Schematic drawing of the two-choice larval assay used in (E).

(C) Schematic drawing of the Flywalk assay used in (F).

(D) Schematic drawing of headspace odor collection equipment for behavioral assays (top figure) and GC-SSR experiments via solid phase micro extraction (SPME, bottom figure).

(E) Larval response indices from the two-choice assay. Green boxes indicate significant differences from the solvent control ($p<0.05$, Wilcoxon signed rank test, n = 10). White line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

(F) Net upwind displacement of mated female flies within 4 s after encountering 500-ms pulses of different fruit headspaces. Orange boxes indicate significantly higher upwind displacement

compared with the negative control hexane ($p<0.05$, Wilcoxon signed rank test, $n = 15$). White line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

RESULTS AND DISCCUSION

Behavioral responses of larval and adult *Drosophila* to the headspace of 34 fruits

We first investigated olfactory behavioral responses by quantifying the chemotaxis of larvae in a binary-choice assay (Figure 1B), and of adult *Drosophila* in the Flywalk assay (Steck et al., 2013; Thoma et al., 2015)(Figure 1C) to headspace extracts from 34 different fruits. These extracts were collected from either ripe fruits or fruits in early fermentation stage according to standard procedures (Figure 1D, see also Experimental Procedures).

We found that larval and adult *Drosophila* displayed significant and differential positive chemotaxis to 31 and 27 of the tested fruit extracts, respectively (Figure 1E-F). Neither larvae nor adult flies showed negative chemotaxis to any of the extracts. The most attractive fruit-headspace extracts for larvae, such as strawberry, passion fruit, and pineapple, elicited either no or mild positive chemotaxis in adult flies. The most attractive headspace extracts for adult flies were red currant and kiwi. In larvae, currant triggered no significant behavioral responses, while kiwi triggered only mild responses. These results clearly indicate that larvae show fruit-specific preferences that differ from those of adult flies.

Coding of fruit volatiles in the entire peripheral olfactory systems of larval and adult *Drosophila*

Having established innate behavioral responses of larval and adult *Drosophila* to the fruit-headspace extracts, we next dissected how these fruit extracts are detected by the complete olfactory systems of larval and adult *Drosophila*. To do this, we performed a system-wide electrophysiological screen from these olfactory systems using the same 34 fruit odors.

We carried out our screen from the 48 Ors of the adult olfactory system by recording from all OSN types and identifying them by using a diagnostic set of odorants (Ebrahim et al., 2015). By doing so, we also recorded from OSNs expressing several ionotropic receptors (Ir) and two coexpressed gustatory receptors (Gr). We furthermore screened the responses of larval OSNs expressing 21 Ors that were previously found to be functional in the adult empty neuron system (Kreher et al., 2005; Mathew et al., 2013). The identification of individual OSN responses in

larvae is almost impossible, as all OSNs are co-localized in a single morphological structure, the dorsal organ. We, therefore, mis-expressed the 11 larval-specific receptors in mutant ab3A OSNs (lacking a functional Or22a) on the adult antenna using the Gal4-UAS system (Dobritsa et al., 2003). For the ensuing analysis recordings from adult neurons expressing the 10 receptors that are expressed both in larval and adult flies were used for the analysis of both developmental stages (for an overview of all neuronal responses see Table S1).

Figure 2

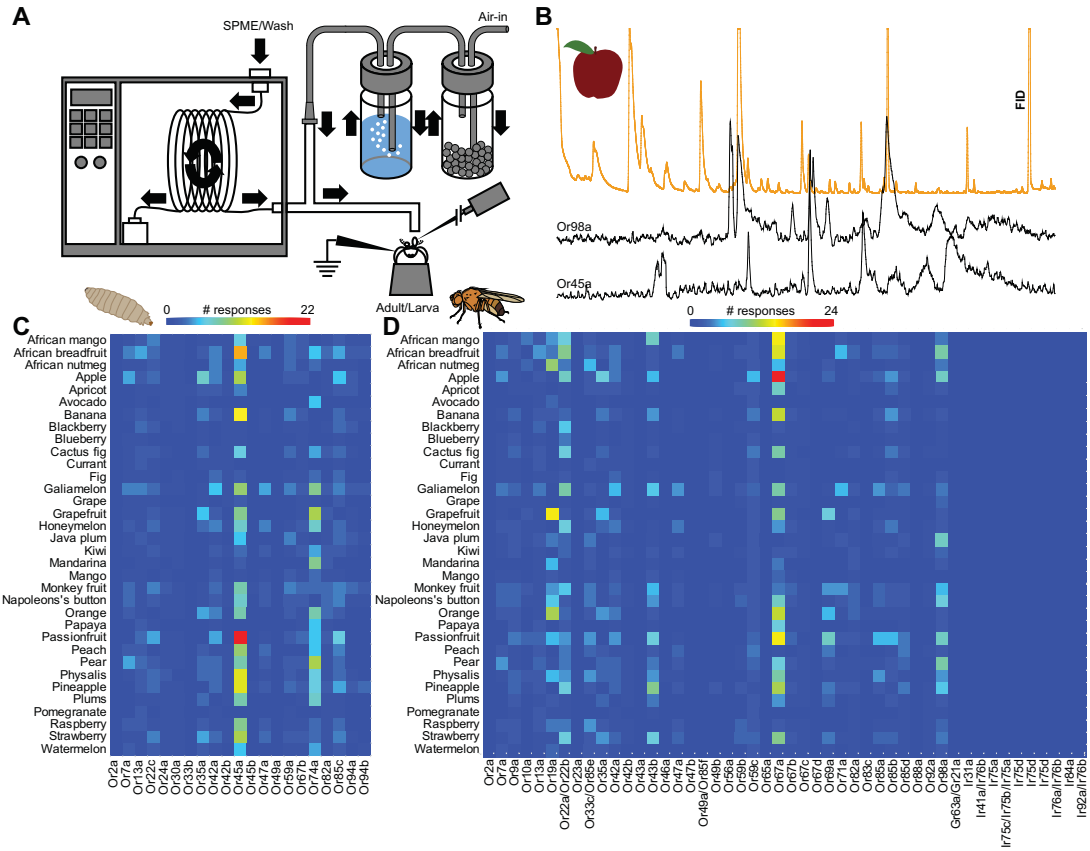


Figure 2. Coding of fruit headspace extracts in the entire peripheral olfactory systems of larvae and adult *Drosophila*.

(A) Schematic drawing of SPME-GC-SSR.

(B) Representative GC-SSR measurement from neurons expressing Or98a or Or45a (black traces) stimulated with headspace extract of apple (orange trace).

(C and D) Heat map of responses elicited by each fruit headspace extracts as determined via a system-wide GC-SSR screen from OSNs expressing the 21 larval Ors (C) and the 48 OSNs expressing Ors, Irs, or Grs of adult flies (D). (n = 3-5).

This system-wide screen was performed using linked gas chromatography-single sensillum recording (GC-SSR) measurements (Wadhams, 1982) (Figure 2A,B). The combination of GC with SSR allowed us to pinpoint the physiologically active flame ionization detection (FID) peaks in each extract and their retention times. Because in some cases the active FID peaks were within the solvent peak, we used linked solid phase microextraction (SPME)-GC-SSR technique to overcome this limitation. SPME is a solvent-free sample preparation method that has been established to analyze plant volatiles (Tholl et al., 2006).

Our screen revealed that the tested fruits elicited 1085 and 1668 excitatory responses from the olfactory systems of larvae and adult flies, respectively (Figure 2C,D). Surprisingly, none of the fruit volatiles elicited inhibitory responses, neither in larval nor in adult flies (data not shown). The number of peaks eliciting responses in single extracts ranged in larvae from 65 as in case of African breadfruit down to seven as in case of blueberry. In adult flies, extracts elicited responses ranging from 101 (passionfruit) down to nine (avocado). At the same time monkey fruit activated the highest number of OSN types characterized by the Or expressed, while avocado activated the lowest number (Figure 2C,D).

Furthermore, our screen revealed that fruit volatiles activated 90% of the screened OSN types in larvae, and 53% in adult flies. 89% of the activated OSN types were activated more than once (Figure 2C and D). In larvae, neurons expressing Or45a, Or74a and Or85c responded to 247 (22.8 %), 147 (13.5%) and 88 (8%), respectively, out of 1085 FID peaks. In adult flies, neurons expressing Or67a, Or22a/Or22b and Or98a responded to 235 (14%), 143 (8.6%) and 124 (7.4%), respectively, out of 1668 FID peaks. Contrary to these obviously rather widely tuned receptors, neurons expressing either Or33b or Or46a were activated only once.

Our screen also revealed that none of the fruit volatiles triggered responses from neurons expressing receptors that are known to signal cues of particular biological importance (Figure 2C,D, Table S1). Neither the ab1C neuron co-expressing the gustatory receptors Gr21a and Gr63a and detecting CO_2 (Kwon et al., 2007), nor the ab10B neuron coexpressing Or49a and

Or85f that governs the fly's avoidance to parasitoid wasps of the genus *Leptopilina* (Ebrahim et al., 2015), nor the ab4B neuron expressing Or56a and signifying the presence of harmful microbes by detecting the key odor geosmin (Stensmyr et al., 2012) were activated by any of the fruit odors. Also, none of the OSNs housed in trichoid sensilla, which are known to detect volatile pheromones (Dweck et al., 2015; van Naters and Carlson, 2007), responded to any of the numerous fruit-derived compounds. Finally, none of the OSNs expressing ionotropic receptors responded to any of the fruit volatiles.

Identification of the active compounds for the screened larval receptors and adult neurons. The active FID peaks from each extract were identified using linked GC-mass spectrometry (MS), linked SPME-GC-MS, and synthetic standards (for an overview of identified compounds in different fruit headspaces see Table S2). The identification of the active compounds was further confirmed physiologically by injecting the synthetic standards in GC-SSR measurements. Based on the retention time, the 1085 and 1668 active FID peaks in larvae and adult flies corresponded to 165 and 278 compounds, respectively. Of these compounds, only 59 and 102 could be unambiguously identified. The other compounds did not produce clear mass spectra and thus, remain unidentified. Fourteen compounds were larval specific, 57 were adult specific, and 45 were common between larva and adult flies (Table S1). Nineteen of these compounds were identified as key ligands for the first time. While in larvae only 10% of the identified compounds activated multiple receptors (e.g. hexyl acetate activated the four receptors Or13a, Or35a, O45a and Or47a), in adults 25% of the identified compounds activated multiple OSNs (e.g. ethyl hexanoate activated seven receptors Or22a/Or22b (ab3A), Or47a (ab5B), Or43b (ab8A), Or69a (ab9A), Or19a (ai2A), Or59c (pb3A), and Or85d (pb3B)).

In order to analyze the chemical space covered by the identified compounds, we next constructed an odor matrix based on 32 optimized molecular descriptors (Haddad et al., 2008) using DRAGON software. The selected descriptors were then subjected to z-score normalization and hierarchical clustering analysis, based on Ward's method.

We found that larval compounds were clustered into three groups (Figure 3A), while adult compounds were clustered into four groups (Figure 3B). The larval groups included one group of 14 aromatics (i.e. benzene containing compounds), another group of 21 aliphatic esters and one aliphatic ketone, and a third group containing seven aliphatic alcohols, nine aliphatic

esters, five aliphatic ketones, and two aliphatic aldehydes. One of the four adult-specific chemical clusters consisted of 23 terpenes, another covered 15 aliphatic esters and two terpenes, while the remaining two consisted of 27 aliphatic esters, eight aliphatic alcohols, four aliphatic ketones and two aldehydes, and of 19 aromatics and two ketones, respectively.

Figure 3

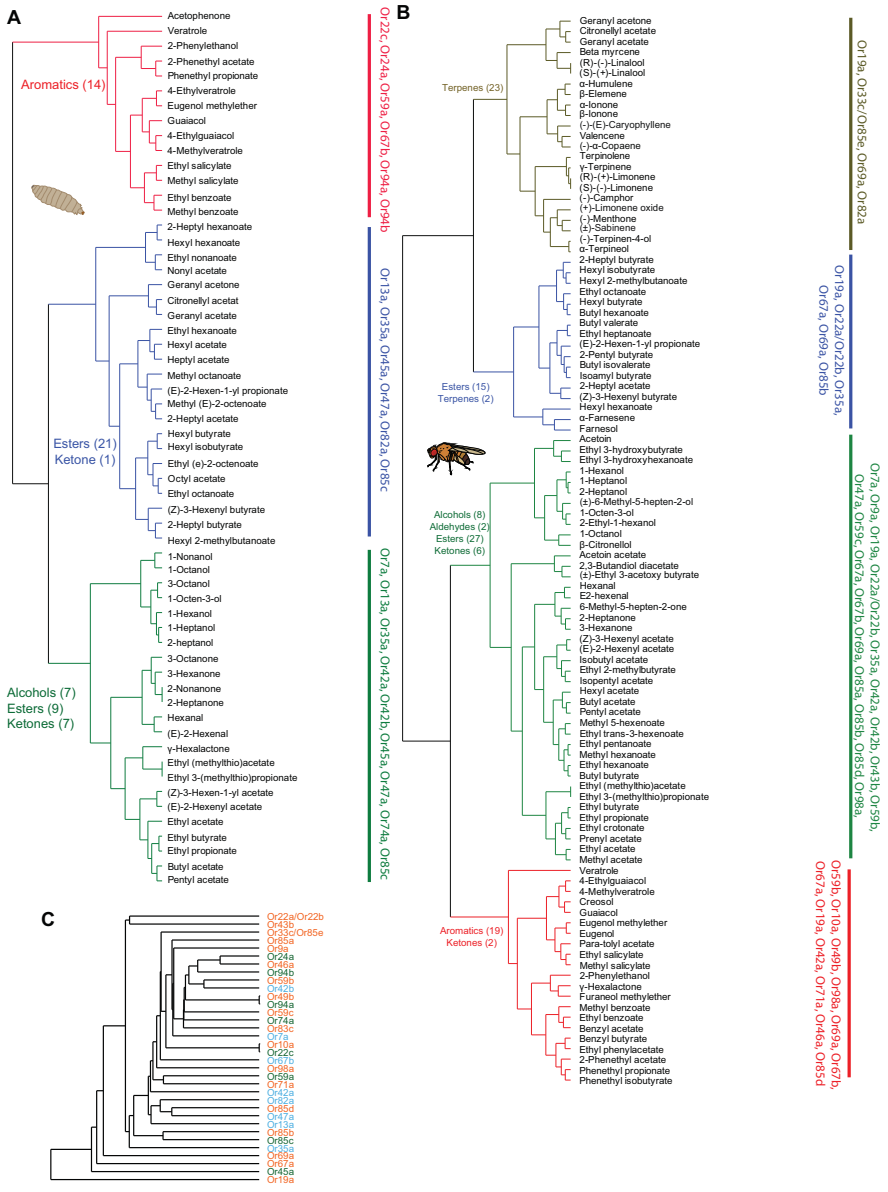


Figure 3. Cluster analysis of compounds detected by larvae (A) and adult flies (B) and of all activated receptors. (A and B) Groups are color coded by chemical classes. (C) Receptors are color coded according to their expression. Green, expressed in larvae; orange, expressed in adults; blue, expressed in both stages. All cluster analyses were performed using Ward's algorithm and Euclidian similarity index.

In both larvae and adult flies, the highest number of responses was observed among aliphatic esters (~ 52 % in larvae and ~ 44 % in adult). In larvae, this was followed by aromatics (~ 24 %), aliphatic alcohols (12%), aliphatic ketone (~8%) and aliphatic aldehydes (~ 3 %), while, in adult flies, it was followed by terpenes (~ 25 %), aromatics (19%), aliphatic alcohols (~ 8 %), aliphatic ketones (~ 6 %), aliphatic aldehydes (~ 2 %).

We noted with interest that adult flies detected 23 terpenes, of which only three were detected by larvae, suggesting that terpenes might be of particular ecological significance to adult flies but not to larvae. We have previously shown that gravid flies prefer terpenes-rich citrus fruits as oviposition substrate and that, in turn, terpenes protect *Drosophila* offspring against endoparasitoid wasps (Dweck et al., 2013).

A cluster analysis of all activated receptors based on their response patterns to the 116 identified compounds in the fruit headspaces (Figure 3C) revealed that the larval receptor Or22c shares its response pattern with the adult Or10a, while the larval Or94b responds to the same compounds as the adult Or49b. Interestingly, while the former two receptors are phylogenetically closely related, the similarly responding Or94b and Or49b show up at very distant places along a phylogenetic tree (Couto et al., 2005; Robertson et al., 2003). Why larvae and adults recruit different receptors for the same function remains open. However, the similar olfactory tuning of distantly related receptors might later lead to a better understanding of ligand receptor interactions.

Correlation of Peripheral Input to Behavioral Output in Larval and Adult *Drosophila*

We finally performed a principal component analysis (PCA) for the 34 fruit odors based on the physiological responses they elicited (Figure 4A and 4B) and compared it with our behavioral screen. Interestingly, we found a correlation of attraction and the first principal component (PC1) in both cases (Figure 4C and 4D). While the PC1 (and therefore attraction)

was mainly (but not exclusively) affected by Or45a in larvae (Figure 4E), the activation of Or67a was the main (but not exclusive) factor in adult flies (Figure 4F). Attraction was positively correlated with neuronal activity in larvae (Figure 4C) and negatively correlated in flies (Figure 4D).

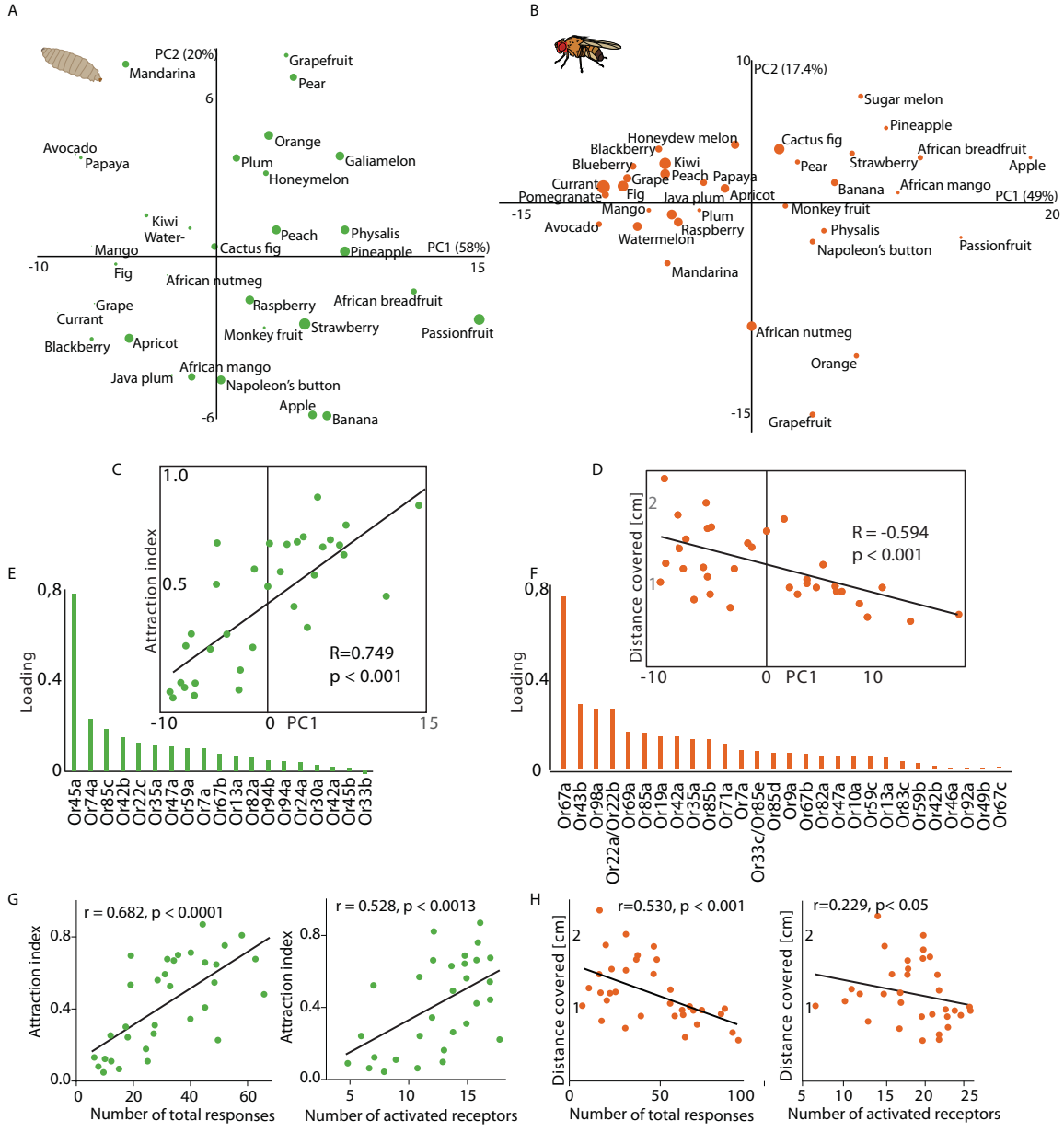


Figure 4. Correlation of Peripheral Input to Behavioral Responses in Larval and Adult *Drosophila*

(Aand B) Principal component analysis (PCA) of all fruit headspaces based on the physiological responses they elicited in receptors of larvae (A) and adult flies (B). Size of dots depicts relative attraction of each fruit headspace in behavioral assays. (C and D) Pearson's correlation of the first principal components from (A and B) and attraction measured in behavioral assays. (E and F) Loadings of the individual receptors for the PCAs shown in (A and B). (G and H) Pearson's correlation of number of total responses (left panel) or activated receptors (right panel) elicited by each tested fruit and attraction measured in bioassays for larvae (G) and adult flies (H).

We gained similar results, when we correlated the total number of either responses or activated OSN types by each tested fruit odor from all 21 larval or 48 adult Ors to behavioral responses elicited by the same fruit odor in both larval and adult flies. We found that in larvae, these correlations were strongly positive (Figure 4G), meaning that as the total number of elicited responses or activated OSNs increases, larvae become more attracted. In adult flies, again, these correlations were weakly negative (Figure 4H).

A positive correlation between OSN activity and attraction in larvae was already suggested by (Kreher et al., 2008), who correlated the sum of the action potentials from all the 21 larval receptors and behavioral responses elicited by a panel of monomolecular odors. Larvae do not have to localize food but grow on the substrate their mother chose to oviposit on. This might be the reason why only few of the 21 receptors seem to govern avoidance behavior (e.g. Or49a governing the avoidance of parasitoid wasps (Ebrahim et al., 2015)). The remaining majority of receptors seems to be involved in detecting positive cues from the fruit, resulting in an overall positive correlation of OSN activity and attraction. Adult flies, however, do need to localize food and oviposition sites. Many of the receptors are tuned to low concentrations, allowing the fly to detect rotten fruits over distance. In addition, female flies need to judge the quality of a potential oviposition site. If e.g. the stage of decay of a fruit is too advanced (which usually goes along with increased emission of odors), harmful bacteria might colonize the fruit and kill the offspring (Stensmyr et al., 2012). The preference for slightly, but not too rotten fruit might explain the negative correlation of OSN activity and attraction in adult flies, as strong OSN activity might signal a too advanced ripening stage. Indeed, it was shown for several monomolecular odorants (Farhan et al., 2013; Strutz et al., 2014) and for the headspace of vinegar (Semmelhack and Wang, 2009) that attractive odors can turn aversive at high concentrations. Based on these

findings one can speculate, that not only the increased emission induced by the ripening process but also the fruit-species related emission rate dictates which fruit is chosen by the fly, with less smelly fruits being preferred.

Obviously, although being equipped with overlapping sets of olfactory receptors, larval and adult flies have evolved different mechanisms for the detection and computation of fruit odors.

SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCEDURES

Fly Stocks

All experiments with wild type (WT) *D. melanogaster* were carried out with the Canton-S strain. halo;Or22a-GAL4/UAS-OrX lines were a gift from John R. Carlson (Yale University).

Headspace Collections

The headspaces of the different samples were collected for 24 h on a Super-Q filter (50 mg, Analytical Research Systems, Inc., www.ars-fla.com). The samples were placed individually in an l liter laboratory glass bottle that was halfway filled with samples and closed with a custom-made polyether ether ketone (PEEK) stopper. Airflow at 0.5 L/min was drawn through the flask by a pressure pump. Filters were eluted with 300 µl hexane and samples stored at -20tC until analysis.

SPME/SPME-GC-SSR/SPME-GC-MS

The samples were placed individually in l0-ml glass vails that were filled with 2-ml of each fruit sample and closed with a cap equipped with a polytetrafluoroethylene-lined silicone septum. After penetrating the septum of the cap with the SPME fiber holder, the SPME fiber coated with 100 um of polydimethylsiloxane (Supelco) was exposed to the headspace in each fruit-containing vial for 10 min at room temperature. Afterward, the SPME fiber was directly inserted into the inlet of a GC connected to either SSR or MS.

GC-SSR/GC-MS

Adult flies were immobilized in pipette tips, and the palps or antennae were placed in a stable position onto a glass coverslip. Sensilla were localized under a binocular at 1000x magnification, and the extracellular signals originating from the ORNs were measured by inserting a tungsten

wire electrode into the base of a sensillum. The reference electrode was inserted into the eye. Signals were amplified (10x; Syntech Universal AC/DC Probe; www.syntech.nl), sampled (10,667 samples/s), and filtered (100–3000 Hz with 50/60-Hz suppression) via a USBIDAC connection to a computer (Syntech). Action potentials were extracted using Syntech Auto Spike 32 software. For GC-SSR, neuron activities were recorded for 1220 s, the time of a single GC run. For GC stimulation, 1 µl of the odor sample was injected into a GC (Agilent 6890, column: DB5, 30 m x 0.32 mm (id), 0.25 µm film thickness; inlet at 250tC, oven: 50tC for 2 min, then 15tC × min^{-1} up to 250tC, held for 5 min; carrier gas: helium, 2.0 ml min^{-1} constant flow). The GC was equipped with a 4-arm effluent splitter (Gerstel, www.gerstel.com), with split ratio 1:1 and N_2 (30.3 kPa) as makeup gas. One arm was connected with the flame ionization detector (FID) of the GC and the other arm introduced into a humidified air stream (200 ml × min^{-1}). GC-separated components were directed toward the palps or the antennae of the mounted fly. Signals from ORNs and FID were recorded simultaneously. Headspace samples were analyzed by GC-MS (Agilent 6890GC & 5975bMS, Agilent Technologies, www.agilent.com).

Chemicals

All odorants tested were purchased from commercial sources (Sigma, http://www.sigma-aldrich.com and TCI America, http://www.tcichemicals.com/en/us/) except for 2-heptyl acetate, 2-heptyl butyrate, 2-heptyl hexanoate and 2,3-butanediol diacetate, which were synthesized in house from commercially available precursors.

Synthesis of 2-heptyl acetate, 2-heptyl butyrate and 2-heptyl hexanoate

2-Heptanol (580 mg, 5 mmol) was dissolved in 15 ml dichloromethane and 10 mmol of the corresponding carboxylic anhydride, 1.4 ml triethylamine and 20 mg of 4-dimethylaminopyridine were added. The mixture was stirred at room temperature for 3-4h, quenched with 20 ml ice-water and extracted with diethylether (3×30 ml). The combined organic layers were washed with water (40 ml) and brine (40 ml), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. Purification with silica gel column chromatography (3:1 to 9:1 hexane/ethyl acetate) yielded racemic 2-heptyl esters as colorless liquids.

Synthesis of 2,3-butanediol diacetate

2,3-Butanediol diacetate was synthesized from 2,3-butanediol with the procedure described for 2-heptyl acetate using 4 molar equivalents of acetic anhydride.

Larval two-choice assay

The larval olfactory two-choice assay is illustrated in Figure 1B. For the measurement of olfactory responses, 50 larvae were briefly dried on a filter paper before being placed in the center of a Petri dish (diameter, 9cm) filled with 1% agarose. The Petri dish contained on one side a filter paper disc (diameter, 0.5cm) loaded with 10 µl of one of the fruit extracts and on the opposite side a similar disc loaded with hexane. After 5 min of larvae placement and covering of the Petri dish, the number of larvae in respective zones was counted and a response index was calculated ((O - C) / T), where O is the number of larvae on the side of the dish containing fruit-headspace extracts, C is the number of larvae on the hexane side, T is the total number of larvae.

FlyWalk Assay

Apart from few technical modifications on the behavioral setup (see below), the FlyWalk experiments were performed and analyzed as described previously (Thoma et al., 2014, 2015)

with 7-d-old mated female flies starved for 24 h before the start of the experiments. In short, 15 individual flies were placed in glass tubes (diameter, 0.8 cm). The glass tubes were aligned in parallel, and flies were monitored continuously by an overhead camera (HD Pro Webcam C920; Logitech). XY positions were recorded automatically at 20 fps using Flywalk Reloaded v1.0 software (Electricidade Em Pó; flywalk.eempo.net). Experiments were performed under red LED light (peak intensity at ! , 630 nm). During the experiments, flies were continuously exposed to a humidified airflow of 20 cm/s (70% relative humidity, 20 °C). Flies were repeatedly presented with 500-ms pulses of various olfactory stimuli at interstimulus intervals of 90 s. Stimuli were added to the continuous airstream and thus travelled through the glass tubes at a constant speed. In brief, 100 uL of each fruit-headspace extract was prepared in 200-uL PCR tubes, which were placed into odor vials made of polyetheretherketone (PEEK). The odor vials were tightly sealed and connected to the stimulus device via ball-stop check valves that allowed only unidirectional airflow through the odor-saturated headspace. Odor stimulation was achieved by switching an airflow otherwise passing through an empty vial (compensatory airflow) to the odor-containing vial. Tracking data were analyzed using custom-written routines programmed in R (www.r-project.org). Flies were assigned to individual glass tubes using the Y-coordinates and thus could be unambiguously identified throughout the whole experiment. As flies were allowed to distribute freely within their glass tubes, they might have encounter the odor pulse at different times. This was compensated for by calculating the time of odor encounter for each individual tracking event based on the X position of the fly, system intrinsic delay, and airspeed. The time of encounter was set to 0, and the speed of movement was interpolated in the interval between 10 s before and 10 s after an encounter at 10 Hz. Because the tracking system does not capture the entire length of the glass tubes, not every fly was tracked for every stimulation cycle, and some entered or left the region of interest during the tracking event; thus, we decided to consider only complete trajectories in the interval between 1 s before and 7 s after odor encounter for further analysis.

AUTHOR CONTRUBUTIONS

H.K.M.D., S.A.M.E., T.R., V.G., J.W., A.S., B.S.H. and M.K. designed all experiments. H.K.M.D., S.A.M.E., T.R., V.G. and J.W. performed all experiments and data analysis. H.K.M.D., B.S.H., and M.K. wrote the manuscript.

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Figure Legends

Figure 1. Behavioral responses of larval and adult *Drosophila* to fruit-headspace extracts.

(A) Clipart of 34 different fruits.

(B) Schematic drawing of the two-choice larval assay used in (E).

(C) Schematic drawing of the Flywalk assay used in (F).

(D) Schematic drawing of headspace odor collection equipment for behavioral assays (top figure) and GC-SSR experiments (bottom figure).

(E) Larval response indices from the two-choice assay. Green boxes indicate significant differences from the solvent control ($p<0.05$, Wilcoxon signed rank test, $n = 10$). White line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

(F) Net upwind displacement of mated female flies within 4 s after encountering 500ms pulses of different fruit headspaces. Orange boxes indicate significantly higher upwind displacement compared with the negative control mineral oil ($p<0.05$, Wilcoxon signed rank test, $n = 15$). White line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

Figure 2. Coding of fruit headspace extracts in the entire peripheral olfactory systems of larvae and adult *Drosophila*.

(A) Schematic drawing of SPME-GC-SSR.

(B) Representative GC-SSR measurement from neurons expressing Or98a or Or45a (black traces) stimulated with headspace extract of apple (orange trace).

(C and D) Heatmap of number of responses elicited by each fruit headspace extracts as determined via a system- wide GC-SSR screen from the 21 larval Ors (C) and the 48 ORNs expressing Ors, Irs, or Grs of adult flies (D). ($n = 3$-5).

Figure 3. Cluster analysis of compounds detected by larvae (A) and adult flies (B) and of all activated receptors. (A and B) groups are color coded by chemical classes. (C) Receptors are color coded regarding their expression. Green, expressed in larvae; orange, expressed in adults; blue, expressed in both stages. All cluster analyses were performed using Ward's algorithm and Euclidian similarity index.

Figure 4. Correlation of Peripheral Input to Behavioral Responses in larval and adult *Drosophila*

(Aand B) Principal component analysis (PCA) of all fruit headspaces based on the physiological responses they elicited in receptors of larvae (A) and adult flies (B). Size of dots depicts relative

attraction of each fruit headspace in behavioral assays. (C and D) Pearson's correlation of the first principal components from (A and B) and attraction measured in behavioral assays. (E and F) Loadings of the individual receptors for the PCAs shown in (A andB). (G and H) Pearson's correlation of number of total responses (left panel) or activated receptors (right panel) elicited by each tested fruit and attraction measured in bioassays for larvae (G) and adult flies (H).

Table S1. List of identified active compounds in both larvae and adult *Drosophila*

Green boxes, detected by larval-specific receptors; brown boxes, detected by adult-specific compounds; blue boxes, detected by receptors expressed both in larvae and adult flies). Compounds in yellow boxes were identified and reported as *Drosophila* ligands for the first time in this screen.

Table S2. List of identified active compounds in 34 fruit headspaces

Red boxes, compound present in this fruit headspace. Compound in yellow boxes were identified and reported as *Drosophila* ligands for the first time in this screen.

General Discussion

This thesis has three main aims. First it tries to sensitize science for the importance of appropriate controls when using the genetic toolbox of *Drosophila melanogaster*. Second and third, it gives new insights in flies´ binary odor mixture processing and in the intraspecific communication of the vinegar fly.

Trust is good, control is better – importance of proper controls

The approach in *Drosophila* of taking mutant flies to examine necessity of receptors works only in systems driven by the principle of one odor – one receptor, like in CO_2 (Jones et al., 2007; Kwon et al., 2007) and geosmin (Stensmyr et al., 2012) or in the pheromone system (Dweck et al., 2015). The characteristics of such systems are narrowly tuned chemosensory receptors, which means they are highly specialized in detecting particular chemical molecules (Grabe et al., 2016). In contrast, most food odor detecting receptors are broadly tuned, which means they are not activated by single odorants, but by a bunch of different chemical molecules (Hallem and Carlson, 2006). Hence, I searched for possibilities to investigate the necessity of single olfactory receptors in different behavioral experimental setups. A quantity of behavioral paradigms – speaking only about *Drosophila melanogaster* - was progressively developed by scientists all over the world. In the beginning of behavioral experiments with *Drosophila*, scientists just observed freely walking flies (Flügge, 1934) until Robert Anholt and colleagues published 1996 the first assay to evaluate flies´

innate odor responses. Since this time the number of paradigms aiming for investigation of *Drosophila* odor responses increased dramatically (e.g. Budick and Dickinson, 2006; Claridge-Chang et al., 2009; Becher et al., 2010; Knaden et al., 2012; Zaninovich et al., 2013; van Breugel and Dickinson, 2014). This scope of possibilities necessitates a good planning and deliberation of experiments, because behavioral results depend very much on the chosen assay. As it is shown in Manuscript I, changing only one parameter affects the results, although testing flies with the same genetic background. We compared a two-choice assay without airflow with the Flywalk (Steck et al., 2012), which is based on odor puffing into continuously flowing air. Although the presented odors were the same in both setups, the experimental flies were challenged in two different ways. While the flies have to follow an odor concentration gradient to find the odor source in the trap assay, in the Flywalk flies need to approach the odor against a wind stream. However, the air flow triggers mechanosensory sensilla (MSS; Todi et al., 2004). The priority objective of MSS on the antennae is auditory perception of acoustic waves (Göpfert and Robert, 2002; Yorozu et al., 2009), but they also detect wind directions. Giving this information, flies do not have to evaluate the odor concentration to find the odor source, they just have to identify the odor and decide whether to go or not to go. From the fly´s point of view the Flywalk represents a much easier challenge in finding the odor source. Furthermore, I could show the mechanics and expression level of the particular effectors play a crucial role regarding their efficiency in behavior. It seems the more complex the mechanism the less efficient are the effectors. While *tetanus toxin* (TeTx) just blocks vesicle release in the neurons and *Kir2.1* just cancels the cells' membrane potential, *reaper* (rpr) and *diphtheria toxin* (*DTA*) work way more complex in the cell.

Both rely strongly on the inner cell machinery, as they induce apoptosis (*rpr*) and inhibit protein synthesis (*DTA*). On one hand there are so many players participating in these two important cell mechanisms that the possibility of side effects with unknown outcome is highly increased. On the other hand, due to so many players being involved, there is a temporal component, as it takes much more time to induce apoptosis respectively inhibit protein synthesis compared to canceling membrane potentials and inhibiting vesicle release.

One step ahead of transgenic flies with silenced neurons are mutant flies lacking expression of the olfactory co-receptor *orco* (Larsson et al., 2004). The most popular hypothesis about the functional mechanism of *orco* is that the co-receptor and the olfactory receptor are not functional on their own, but form a dimer which can bind odor molecules and activate the neuron (Wicher et al., 2008). Therefore, *orco* seems to be crucial for the flies' olfaction. However, in a former publication of our department *orco*-mutant flies still responded to some odors (including vinegar) in Flywalk experiments (Steck et al. 2012). It is important to know that the olfactory system of *Drosophila* comprises olfactory receptors (ORs), a single gustatory receptor (GR) and ionotropic glutamate receptors (IRs) responding to volatiles. The latter have been described to detect mainly amines and acids **(**Silbering et al., 2011**)**, which are highly present in vinegar. Up to date it is not known if or how information coming from ORs into the antennal lobe alters the information transferred from IRs. During our experiments we observed a switch in olfactory-guided behavior between wild type and *orco*-mutants as well as in flies coexpressing *TeTx* with *orco*. While wild type flies behaved neutral (neither attracted, nor repelled) towards trans-2-hexenol (t2H), *orco* mutant flies

and those expressing *TeTX* were highly attracted by this odor (Manuscript I). I hypothesize based on these results, that signals coming from ORs indeed influence the information transmitted by neurons expressing IRs or GRs. Although *orco* mutant flies and those, where *TeTX* was coexpressed with *orco* exhibited similar behavior in the Flywalk experiments, their performance in an open-field arena was strikingly different. While flies expressing *TeTx* were not able to locate the odor source in the middle of the arena, *orco*-mutant flies were still capable of doing so. One explanation for this might be the different histories of both lines: The *TeTX* flies emerged one week before the experiment and due to the crossing procedure were the very first generation with non-functional *orco*-expressing OSNs. At the same time, *orco*-mutants are more than 10 years in laboratory use, which equals more than 330 fly generations giving a generation time of two weeks. For comparison, the calculated most recent common ancestor of humans lived only a few thousand years ago (Rohde et al., 2004). A generous estimation of a generation time of 30 years leads to only 77 generations. Although flies living under laboratory conditions do not underlie a high evolutionary pressure, 330 generations without functional *orco* could have led to the establishment of alternative olfactory channels (e.g. IRs) to compensate for the missing information. This could explain why – contrary to *TeTX* flies – *orco*-mutant flies are not only capable of just identifying the odor, but can also track down an odor gradient. Recently, Brown et al. (2017) found evidence for such a guided selection in *Drosophila* within only 30 generations. The authors created different subpopulation by selecting flies with same odor-guided behavior and investigated their differences in feeding behavior. At the end the scientist found 91 genes

altered in their expression levels within the subpopulations compared to non-selected control strains.

"Survival of the fittest" is often mistaken that the strongest individual or population will survive, but it is the most adapted one that will succeed. The term was first stated by Herbert Spencer 1864. Only later Charles Darwin used this term in the fifth edition of his well-known book "Origin of the species" (first edition: 1859). In the following paragraph I will describe an example, how *Drosophila* has adapted its intraspecific communication system.

A new way flies communicate and how this is modified by others

Since a long time, scientists have studied fly pheromones (Shorey, 1973; Ronderos et al, 2014; Dweck et al, 2015; Dekker et al., 2015). In this thesis I contributed to two studies deepening our knowledge about the pheromone system in *Drosophila melanogaster*. We could show that flies attract each other by depositing frass spots on suitable food sources (Manuscript II). These fecal droplets contain compounds predominantly detected by the pheromone receptors OR47b, OR67d and OR88a. OR47b and OR88a were described by Dweck et al. (2015) to detect methyl laurate (ML), methyl myristate (MM) and methyl palmitate (MP), while OR67d is known as the receptor for the male sex pheromone 11-*cis*-vaccenyl acetate (cVA; Brieger and Butterworth, 1970; Kurtovic et al., 2007). In addition, it was shown that several fatty acids also found in the GC-MS profile of frass activate OR47b (Lin et al., 2016). Putting all this information together, I hypothesize that *Drosophila frass* spotson food sources work, due to their higher amount of pheromones compared to the

flies´body, as an amplifier in the fly´s pheromone system to transduce information not only on close range (< 10 cm), but spread it further. Hence, fecal droplets may have several functions in intra- and interspecific communication. Male feces contain cVA, which is known to have an anti-aphrodisiac effect in *D. melanogaster* males` mating behavior (Zawistowski and Richmond, 1986; Auer and Benton, 2016), while for females it is an aphrodisiac, as well as an aggregation signal. On the opposite, female feces contain a high amount of (7Z-11Z)-heptacosadiene and (7Z-11Z)-nonacosadiene, which inhibit mating between different fly species (Fan et al., 2013) and support aggregation behavior. So we conclude that dropping single fecal spots works as a general aggregation signal in *Drosophila melanogaster*. This theory is supported by the fact that the GC-MS traces of adult frass and cuticular hydrocarbons (CHCs) are identical. Giving this information it is coherent that *Drosophila melanogaster* is capable of discriminating frass of closely related species from those of non-related species. Hence, I should note, that the feces from closely related species elicited a similar attraction behavior in Flywalk experiments. Thus we conclude that a precise identification needs additional information not provided by fecal spots. However, the aggregation of individuals *via* feces is also known from other species. Bark beetles (Symonds and Gitau-Clarke, 2016) and German cockroaches (Wada-Katsumata et al., 2015) use fecal droplets as a pheromone signal to conspecifics. Furthermore, feces were already used as a source to identify novel pheromone compounds (Bellas et al., 1969; Tumlinson et al., 1969; Hall et al., 2002). Using this approach in *Drosophilids* could help to establish new ways of pest control. Over the last couple of years *Drosophila suzukii* became a sweeping pest for fresh fruits in the US as well as in Asia and

Europe (dos Santos et al., 2017). Knowing the flies' pheromone could allow the development of efficient traps and the use of mating disruption, which is already used to control *Ostrinia nubilalis*, the European corn borer moth (Baker et al., 2016).

Due to the fact that we were the first publishing attraction behavior of flies towards conspecific feces it is not known if pheromones are the one and only source of attraction in the flies´ frass. If we take a look around in the animal kingdom we find another possibility which could contribute to the observed behavior: gut bacteria. A symbiosis of bacteria and animals is conserved over species and taxa (Tsuchida et al., 2017; Zhang et al., 2017; Qi et al., 2017). Humans also have a so called gut flora (Guarner and Malagelada, 2003), which means that our gut hosts millions of different bacteria species helping us to digest aliment and resorb important nutrients. Thus, it is most likely that feces of flies contain besides already found pheromones also microorganisms like bacteria or yeast. Especially yeast would be a potential candidate to elicit attraction behavior in *Drosophila melanogaster* since Becher et al. (2012) found evidence that yeast and not the fermenting fruit itself attracts flies to a food source.

Not only mutual bacteria live with animals. In humans and other animals, harmful bacteria, so called pathogens, often cause severe diseases like dysentery (*Shigella*; Mattock and Blocker, 2017), anthrax (*Bacillus anthracis*; Cromartie et al., 1947) or tuberculosis (*Mycobacterium tuberculosis*; isolated by Robert Koch 1882; Barberis et al., 2017). Also *Drosophila* encounters several pathogens in nature. In Manuscript III we tested the impact of three of them on the flies' survival and behavior: *Serratia marcescens*, *Pseudomonas entomophila* and *Erwinia carotovora carotovora*. While the first two are known for

infecting different insect species and causing septicemia (Flyg et al., 1980; Burritt et al., 2016) respectively massive destruction of gut cells (Vodovar et al., 2005), *E. carotovora carotovora* is a phytopathogen in the first place which also induces an immune response in its vector *Drosophila melanogaster* (Basset et al., 2000). For comparison we tested also three bacteria species, which are known for not inducing any immune response in flies. *Lactobacillus plantarum* is found in fermented food products and anaerobic plant matter (Pederson, 1936), while *Acetobacter pomorum* was first isolated from industrial vinegar fermentations (Sokollek et al., 1998). Beside these two we also tested the world´s most common parasitic microbe – *Wolbachia*. Even though these bacteria evolved over the last decades from parasites to mutualists in some insects (Weeks et al., 2007; Bhattacharya et al., 2017). Today *Wolbachia* is used in disease control by infecting mosquitoes, which are vectors for severe diseases like dengue fever (Lopes Silva et al., 2017) or malaria (Zélé et al., 2017).

In this study we examined, whether infection with bacterial pathogens alters the pheromone profile of *Drosophila melanogaster*. Therefore, we infected Canton S wild type flies with the mentioned bacteria. Interestingly we found an increase of adult flies` body odorants only in those flies which were infected pathogens that induce immune responses. This alteration of 12 compounds including ML, MM and MP, i.e. recently identified fly pheromones (Dweck et al., 2015). Further we could show with our experiments that the increase in pheromone production leads to a higher attraction of conspecifics. This makes sense if we consider the ecological big picture of this phenomenon. The pathogens profit from the increased pheromone production of the flies, because the enhanced attraction and the

resulting higher density of flies helps spreading of the microbes (Diaz and Restif, 2014). Despite the increased attraction of infected flies, we did not observe any increased mating. Therefore, the increase of pheromone production does not seem to be of any benefit for the flies. But why are only bacteria inducing an immune response using this method to enhance their own spreading? This could be due to the immune and metabolic systems of the host. Testing flies with a reduced immune response we could show that the immune system itself leads to the increase of fatty-acid pheromones production, whereas an artificial activation of the immune system or infecting flies with heat-killed bacteria could not induce an increase in the flies` pheromone profile. Taken together, activating the host´s immune system is necessary but not sufficient to induce a higher pheromone production during infection in flies, which suggests a complex mechanism. However, also the flies' hormonal system and metabolic system are known to be affected by bacterial infection (Diangelo et al., 2009). Further a linkage between the immune system, insulin signaling and the fat body in the flies' brain was shown (Chambers et al., 2012). Since the altered pheromones (ML, MM and MP) are fatty acids, we used different approaches to investigate from which of the two pathways they derive. It has been shown that males´ mating success increases with age (Markow and O´Grady, 2008), suggesting an increase of juvenile hormone. Further Lin et al (2016) brought evidence that this hormone is involved in sensitization of OR47b, the receptor for MP. Hence, we tested transgenic flies deficient in producing juvenile hormone and found a decrease in pheromone abundance. Second we used RNAi against FOXO, a transcription factor involved in insulin signaling (Barthels et al., 2005). We could not use transgenic flies, because of lethal effects of FOXO- deficiency. This

already shows the broad effect of insulin signaling (e.g. oocyte growth and maturation; Das and Arur, 2017). Further we investigated the effect of p38, a stress response regulator, but since it directly phosphorylates FOXO (Asada et al., 2007) both p38 and FOXO are not independent path ways. Although we found again a decrease in pheromone level, we cannot precisely locate their source, due to the broad involvement of the insulin pathway. I conclude that both the hormonal and metabolic pathways have an influence on the pheromone production, but there may be other contributing factors since the pheromones were only decreased, but not vanished.

Behavioral responses to binary mixtures and their processing

Up to this point I highlighted the complexity of the flies´ pheromone system. Now I want to focus on the complementary part of insect olfaction – detection of food odors. This field is complex too, as in nature flies are surrounded by thousands of different odor sources, all having a different meaning to the insects. Furthermore, odorants do not occur separated, but most likely in complicated odor blends. Flies, to exemplify insects, detect these mixtures during flight (long distance, Becher et al., 2010) or while walking (short distance, Flügge, 1934; Steck et al, 2012) and have to extract the information and decide whether to go for the source or not, within seconds. General food odors are coded by the combinatorial code, which means, that several OSN populations, represented by glomeruli in the AL, are activated and this activation pattern leads to behavioral output. Odors with a special ecological meaning are coded *via* so called labelled lines. These odors are not coded

through a pattern in the AL, but activate only a single glomerulus, whose information is unmodified transferred to higher brain centers (e.g. iridomyrmecin or geosmin). Both odors are signaling danger for *Drosophila*. While geosmin is emitted by harmful mold occupying a potential food source (Stensmyr et al., 2012), iridomyrmecin is a pheromone of the parasitic wasp family *Leptopilina* (Ebrahim et al., 2015).

In manuscript IV we break the natural complexity down to binary mixtures of odors with an opposed valence. A former study of *Drosophila melanogaster* behavior already showed a repellent-specific reduction of attractiveness of binary mixtures compared to the attractive compound alone (Thoma et al., 2014). In general, we were able to confirm these results, but also found that this reduction strongly depended on the ratio of the attractants and repellents in the mixture.

There are two potential ways of coding blends in the antennal lobe: first possibility is elemental coding, where single parts of a whole blend are enough to identify the source. The other one is called configural coding, which means that compounds are integrated into a unique perceptual whole. Good examples are found in the human visual system. While the recognition of a face is done by configural coding (Kanwisher et al., 1997), we are also able to just look at the mouth and know it belongs to a face (elemental coding; Whalen et al., 2004). However, behavioral responses of flies to odor mixtures can be calculated in the antennal lobe as the sum glomeruli activated by the single compounds (elemental coding) or by recruitment of an additional network (configural coding). As we found that both single compounds and mixtures activate the same glomeruli, we conclude an elemental coding within in the antennal lobe.

However, during behavioral experiments we found that flies responded to binary mixtures with a higher concentration of the attractant in the same way as to the attractant alone and with decreasing the concentration of the attractant within the mixture, the attractiveness of the mixture decreased. It was shown in a former study that mixture compounds affect the perception of each other (Silbering and Galizia, 2007). Furthermore, another study highlighted that OSN-PN synapses do not work linearly (Kazama and Wilson, 2008). A higher odor concentration increases the vesicle release probability of the presynapse, thus it increases the threshold for effects of lateral inhibition (Olsen and Wilson, 2008). We saw no effect of inhibition to the baseline activity of attractant glomeruli when presenting the repellent alone. This could be due to a low spontaneous activity of the corresponding OSNs. Summarizing this knowledge so far we conclude that the strength of OSN activation determines the strength of PN activation, which again leads to differences in lateral inhibition efficiency (Hong and Wilson, 2015; Seki et al., 2017). In addition, lateral inhibition seems to be dependent on the ratio between mixture compounds. Keeping the concentration of the attractant constant while lowering the repellents´ concentration resulted in no inhibition. Reducing again the attractant and lowering the repellent brought lateral inhibition back. So we can conclude that this effect depends strongly on the ratio between the players.

The described mechanism of lateral inhibition is true for all regular odors, but there are some special odors with a certain ecological meaning in a fly´s life. These exceptions are called labelled lines. They are defined by an odor activating only a single OR type, whose information reaches the higher brain centers unmodified (e.g. pheromones or geosmin; Stensmyr et al.,

2012; Dweck et al., 2015). An example for a repellent labelled line in *Drosophila melanogaster* has been described for the detection of a harmful mold *Penicillium spec*. These microorganisms grow on rotten fruits and are life-threatening for *Drosophila*. The mold emits a special odor (geosmin; Stensmyr et al., 2012), which is only detected by OR56a in the vinegar fly eliciting a direct avoidance response. In our lateral inhibition experiments, however, we exposed flies either to an ecological relevant binary mixture of balsamic vinegar and geosmin or to a mixture of balsamic vinegar and another well-described repellent (Knaden et al. 2012) benzaldehyde. While we observed again lateral inhibition and reduced attractiveness when testing latter mixture, there was no physiological inhibition found with geosmin, although flies showed reduced attraction to the mixture in behavioral experiments. Searching for an explanation of this phenomenon, we have to take a look on another repellent labelled line in *Drosophila* – carbon dioxide (CO_2). In a study from 2015, Lewis et al. showed that balsamic vinegar and CO_2 do not interact on the antennal lobe level, but on the mushroom body level. Since both, geosmin and CO_2, are labelled lines, it is possible that both cues are processed in a similar way. Considering the behavioral response of the flies to those binary mixtures as innate, it is also possible to take a brain area in account, which is known for its important role in innate behavior – the lateral horn (Fisek and Wilson, 2014; Seki et al., 2017). Today it is known that the lateral horn is separated into functional areas regarding the valence of an odor (Strutz et al., 2014). Due to this compartmentalization a possible implementation of responses to mixtures containing labelled lines may also occur in the lateral horn. Further it could be shown, that the more PNs leaving a glomerulus, the less LN innervation occurs. Interestingly, labelled lines (e.g. OR56a

– geosmin), beside pheromone glomeruli, have the highest number of PNs in their corresponding glomeruli (DA2; Grabe et al., 2016).

The question that rises at this point is: What is lateral inhibition good for? Why does it happen? Lateral inhibition seems to have several functions for the perception and processing of sensory input. A recent study raised the hypothesis that lateral inhibition works during nociception in our skin enabling our brain to localize the source of pain. Lacking this mechanism may lead to radiation in some chronic types of pain (Quevedo et al., 2017). Furthermore, lateral inhibition is involved in processing of visual cues. In addition to the known and established function of sharpening edges of different shades of dark and light colors (Kolb, 2003), a new role of lateral inhibition was proposed in 2016: According to physiological and clinical observations it is hypothesized that lateral inhibition is also responsible for reversed colors we see in afterimages (Jerath et al., 2016). Regarding the insect world and here especially the olfaction of *Drosophila melanogaster*, four general functions were postulated for lateral inhibition over the last decade: gain control (Olsen and Wilson, 2008; Root et al., 2008), making PN responses more transient (Olsen et al., 2010), coordination of synchronous oscillation among PNs (Tanaka et al., 2009), and further contribution to mixture interactions between two odors (Olsen et al., 2007; Silbering and Galizia, 2007). Lateral inhibition in the antennal lobe of *Drosophila* mainly involves GABA (Ng et al., 2002; Wilson and Laurent, 2005), glutamate (Liu and Wilson, 2013), tachykinin (Ignell et al, 2009) and other neuropeptides (Carlsson et al., 2010). However, in our study we concentrated prior on GABAergic inhibition. It is worth to mention that GABAergic inhibition is

predominantly presynaptic (Nagel et al., 2015). Interestingly both, OSN axon terminals but also PNs, are inhibited *via* $GABA_A$ and $GABA_B$ receptors (Root et al., 2008; Wilson and Laurent, 2005). Both receptor types differ in function and in the time they are activated. While ionotropic $GABA_A$ receptors respond fast and mediate inhibition in early response phase, the slower metabotropic $GABA_B$ receptors transmit inhibition in the later phase of the response (Olsen and Wilson, 2008; Wilson and Laurent, 2005). In general, there are three different mechanisms of lateral inhibition: First OSNs housed in the same sensillum can affect each other directly *via* ephaptic coupling, which means that the neurons affect the extracellular electric field within the sensillum lymph (Su et al., 2011; Su et al., 2012). The second possibility, I already mentioned, is inhibition on synaptic level in the antennal lobe (Olsen et al., 2010; Silbering and Galizia, 2007). Finally there could be either a global inhibtion of all glomeruli or a glomerulus specific lateral excitation of PNs (Olsen et al., 2007). Our experiments show that the lateral inhibition effect we observed in the antennal lobe of *Drosophila* occurred especially on the synaptic level. Using different antagonists for GABA receptors we were able to refine this mechanism and found that different attractant glomeruli are differently inhibited by the activity repellent-specific glomeruli. I will explain this phenomenon using the binary mixture of balsamic vinegar and benzaldehyde as an example. Balsamic vinegar activates seven glomeruli, whereof four are affected by lateral inhibition through activation of glomeruli by the repellent benzaldehyde. This inhibition can be mediated presynaptic *via* $GABA_B$ receptors or postsynaptic *via* $GABA_A$ receptors. Up to date it was thought that $GABA_A$ receptors contribute only weakly to inhibition effects (Wilson and Laurent, 2005). Although we could show that they have actually a

major role during inhibition (manuscript IV), it is still not known why different glomeruli use different inhibition strategies. A possible hypothesis is that this phenomenon is connected to special characteristics (e.g. different number of PNs, different volume; Grabe et al., 2016) of single glomeruli. Furthermore, although activated by the same odor and getting the same amount of GABA, some glomeruli are stronger inhibited than others (Hong and Wilson, 2015).

Beside the lack of clarity about the concrete mechanism behind inhibition it is also under debate if LNs in the antennal lobe work globally or more glomeruli specific. There are contradictory studies about equal global release of GABA from LNs (Hong and Wilson, 2015) and a GABA release dependent on glomeruli (Ng et al., 2002). First of all, it is worth to mention that the morphology of LNs is consistent with both theories, because most LNs innervate most glomeruli, but there are some exceptions with LNs innervating only a small subset of glomeruli (Das et al., 2008; Chou et al., 2010; Seki et al., 2010). In our study we could observe vanishing lateral inhibition by silencing GABA synthesis using RNAi against GAD1, a gene coding for a key enzyme in GABA biosynthesis (Ng et al., 2002). Hence, with our data we support the theory of global release of GABA. Looking at the big picture, it seems logic to use a global GABA release, because this leads to global inhibition and ends in general gain control for incoming signals. Although we could show the major role of globally released GABA, we found also specific interactions between glomeruli using artificial activation and silencing methods on single OSN populations. In our case we found that two glomeruli, DL1 and DL5, activated by the used repellent have a certain impact on glomeruli activated by the mixture´s attractant. While

DL1 induces inhibition in DM1 and DM4, DL5 affects strongly the activity of DM3, but only little of DM2. Furthermore, the manipulation of DL1 had a striking impact on flies´ attraction behavior. This corresponds with data shown in the past, demonstrating the great meaning of OR42b (DM1) and OR59b (DM4) for attraction of flies towards single attractants (Semmelhack et al., 2009; Knaden et al., 2012).

I hypothesize that flies use elemental coding for detecting potential food sources. This way even complex odor blends, like those deriving from fruits may are broke down to single key odors, which mediate attraction. To test this, in the last manuscript we examined the peripheral olfactory system of *Drosophila* adults and larvae, and tested full blends and single compounds in electrophysiological and behavioral setups.

Different food preferences in larval and adult flies

It is an old question for human teenagers if mother always knows best. We abstracted this question and transferred it to *Drosophila melanogaster*. In our study (Manuscript V) we examined behavioral responses of *Drosophila* larvae and adults to 34 different fruits fitting the ecological niche these insects fill in. Further we highlighted how many olfactory receptors detecting fruit volatiles in both developmental stages. It is worth to note that larvae have only 21 OSN types expressing in total 25 different ORs housed in the dorsal organ (Kreher et al., 2005). In contrast adults have 50 OSN types expressing 37 ORs, 12 IRs and GR21a in antennae and maxillary palps (Jones et al., 2007; Grabe et al., 2016). However, larvae and adult flies share a set of 13 ORs. Hence, one could expect not a congruent but similar behavior of larvae and adult flies in response to

different fruit headspaces. In fact, we could not find any similarity in fruit preferences, but neither larvae nor adults showed any avoidance behavior when exposed to fruit headspaces. Recently Lihoreau et al. (2016) found evidence that adult females and larvae have different needs for food. While females choose their own food source depending on their inner status, they showed a preference in laying their eggs in a substrate with more carbohydrates. Giving the fact that larvae grow best on high-carb/low-protein food, but then had a disadvantage in survival and learning, Lihoreau et al. concluded that they need a balanced food containing carbohydrates as well as proteins. Further it was shown that larvae and adult flies of *Drosophila melanogaster*, both feeding on yeast (Hoang et al., 2015), have different preferences concerning the yeast that is present in the food (Anagnostou et al., 2010).

In the beginning of this section I mentioned, that we not only observed behavior of larvae and adults but also measured receptor activation by fruit volatiles. We found that the single odorants activated 90% of the larval OSNs and 53% of the adult OSNs. When the activation of ORs was correlated with the behavioral output, we found a positive correlation in larvae, but a negative one in adults. This means the more ORs activated in dorsal organ, the more attractive is the received odor for the larva. The opposite is true for adults: the more ORs become activated, the less attractive is an odor blend for the fly. In general, adults detected a broader set of terpenes (23 compared to three in larvae). Terpenes naturally occur in citrus fruits, which were shown as preferred oviposition sites of *Drosophila* and are avoided by flies` parasitic wasps (Dweck et al., 2013). This is not the first publication about insects using terpenes to defend themselves against enemies. Nutting et al. (1974)

showed that soldiers, but not workers, of the North American termite *Tenuirostritemes Tenuirostris* produce α-pinene, myrcene and limonene in a frontal gland, which are both an alarm pheromone and insecticidal.

Taking together and to answer the question at the beginning of this section: Mothers may not always fulfill the preference of their offspring, because they have to balance nutrition and protection to guarantee larval survival. So mother indeed knows best.

Perspective and future questions

With this dissertation I could show that even small insects as *Drosophila melanogaster* have a more complex pheromone system, than originally expected (Manuscript II and III). Using the knowledge gained especially in Manuscript II, future projects could be set to investigate pest species like *Drosophila suzukii* (Keesey et al., 2015). Since these flies lack *cis*-vaccenyl acetate (cVA) as a pheromone (Dekker et al., 2015), it is important to reveal their species specific sexual communication system. Finding such a key player as cVA could lead to ecological pest control methods, e. g. fly traps or mating disruption. Another possible strategy for pest control is making use of push-pull-technique for flies. This means a source of repellence (either naturally or artificial) is placed among the economic plants to push the flies away and a source of attraction surrounds the field to pull the flies there. This system is already used to get control over the Tsetse fly, vector of *Trympanosoma* pathogen (sleeping desease), in Kenya. Cows in small holder farms are protected from the flies wearing

a collar containing repellent compounds from a non-host waterbuck. Additionally targets covered with attractants (cow urine and acetone) leading the insects into traps are placed nearby (Saini et al., 2017).

Also the results of Manuscript III could be potentially used for pest control. As microorganisms can be manipulated by genetic engineering, one could think of designing bacteria competent to increase the pheromone level of insects, but in the same moment sterilizing infected individuals to control population size. Not genetically modified, but natural occurring bacteria are already used in Australia and Brazil to neutralize the danger of infection in mosquitoes transferring pathogens of dengue fever (Asad et al., 2017) and Zika virus (Caragata et al., 2016). Combined with bacteria increasing pheromone levels, this method could become a self-selling item of ecological disease control. Another ecological way of a pest control project could be based on the data shown in Manuscript V, although many following experiments are needed to fully understand its meaning – especially bringing the laboratory data to the field. However, knowing females balance their own food preference and best sources for larval development, one could protect harvest by planting other plants around, which have a higher preference index in female flies (intercropping; Hata et al., 2016).

Speaking of bringing laboratory data to the field: Once more (Thum et al., 2006), I highlighted the importance of appropriate and extensive controls in laboratory experiments, especially when it comes to genetics (Manuscript I). Each organism is a complex building of at least hundreds of genes, which encode for proteins with a distinct function. If we manipulate one gene, we expect a major function, and in my case impaired behavior. The truth is that we cannot be sure if our manipulation has only

this one major effect, or if there is suddenly a chain reaction causing other impairments. Further, if we want to check a certain gene for its function by silencing or artificial activation we have to consider a certain variability of expression strength in different cell types (Natarajan et al., 2012).

Summary

There are numberless different animal species living on this planet. The biggest subgroup is represented by insects with circa six million species. They continuously send and receive information to orientate in their environment and communicate among each other. In this way insects localize food sources, avoid predators, find mating partners and at last find suitable oviposition sites. As the world is loaded with a cacophony information, insects use different channels to extract the important information from a noisy background. Depending on their environment and their niche, insects rely mainly either on visual or olfactory cues – or sometimes on both. Additional information can be gathered by mechanosensation, vibration or gustation.

The main goal of this dissertation was to highlight the contribution of single olfactory receptors (ORs) to olfactory-guided behavior in *Drosophila melanogaster*. One core area I focused on is the sexual intraspecific communication in these flies. Hence, I and my coworkers found a so far unknown source of fly pheromones – feces (Manuscript II). Among other methods I used the Flywalk – a high-throughput bioassay developed in our department – to show that *Drosophila melanogaster* is able to detect feces´ headspace and response with attraction towards it. Additional research highlighted the similarity of the chemical profiles of the frass of flies and their cuticle. Hence, we found methyl laurate, methyl myristate, methyl palmitate and 11-*cis*-vaccenyl acetate (cVA), which were previously described as fly pheromones. In addition we confirmed that the flies´ attraction towards feces was gone, if we silenced single olfactory sensory neuron (OSN) populations known for their

receptivity for one of the mentioned pheromones. Further we could show exploitation of this sensitive system by parasitic bacteria (Manuscript III). Pathogens, activating the flies´ immune system, are able to increase the amount of pheromones produced by the host. Thus it comes to enhanced contacts between flies resulting in a higher probability for the bacteria to spread.

In the second part of my dissertation I dealt with another part of the insects´ olfactory system – perception and processing of food odors. Here my colleagues and I first investigated if *Drosophila* larvae and adults show the same preferences for different fruits (Manuscript V). Indeed we found big differences in food preferences between these two developmental stages. While larvae choose strawberry, passion fruit and pineapple, adult flies go for currant, kiwi and fig. We could also show that adult flies preferred those fruit odors that activated few OSN populations, while larvae were mainly attracted when many OSN populations were strongly activated. This might reflect the different habitats adults and larvae live in, with larvae being always exposed to very high odor concentrations while digging in and feeding from rotten fruit. As a first step to understand the mechanism behind this I investigated the processing of food odor mixtures (Manuscript IV). Since fruit odors are basically a blend of several odors, we kept it simple for experiments and used binary mixtures of attractive and aversive odors. Thus we found a ratio dependence response of the tested animals, which means a high concentration of the attractant results in flies´ attraction towards the mixture and *vice versa*. In this connection we also investigated the role of local interneurons (LNs) in the antennal lobe and their contribution in signal modulation. Interestingly, we found glomeruli specific GABAergic

inhibition patterns. To our knowledge we found for the first time that some tested glomeruli were both pre- and postsynaptic GABAergic inhibited, while others showed only postsynaptic inhibition by GABA.

Finally, I was able to show differences in efficiency of a set of neuronal silencers depending on the used bioassay. While *reaper* and *diphtheria toxin* could not abolish odor-guided behavior in flies in any experimental setup, the efficiency of *tetanus toxin* and the potassium channel *Kir2.1* was highly depending on the expression level, as well as on the bioassay (e.g. with or without air flow). At the end homozygous expressed *tetanus toxin* was the only tested effector capable of fully mimicking the *orco*-mutant phenotype.

This result highlights the importance to make a careful use of the fascinating *Drosophila* neurogenetic toolbox and to always consider the behavioral tasks an animal has to fulfill when interpreting the results.

In general, by better understanding insect olfactory attraction this dissertation opens the door to a future where e.g. feces of pest species could become analyzed to find attractants and to generate new integrated pest management strategies. Thus we could protect not only our harvest, but also the environment – mother earth.

Zusammenfassung

Auf diesem Planeten leben unzählige Tierarten, von denen Insekten mit ca. sechs Millionen Arten die größte Untergruppe bilden. Tiere senden und empfangen ständig Informationen, um sich in ihrer Umgebung zu orientieren und mit anderen zu kommunizieren. Auf diese Weise finden Insekten Nahrung, vermeiden potentielle Fressfeinde, finden Paarungspartner und nicht zuletzt geeignete Eiablageplätze. Dabei benutzen sie verschiedene Kanäle, um die entsprechenden Informationen aus der Umgebung herauszufiltern. Abhängig von der Umgebung und der Nische, die eine Art besetzt, verlassen sich Insekten hauptsächlich entweder auf visuelle oder olfaktorische Reize – in manchen Fällen auch auf beides gleichzeitig. Zusätzliche Informationen sammeln sie über Mechanorezeption, Vibration oder den Geschmackssinn.

Das Hauptziel dieser Dissertation war es, den Beitrag von einzelnen olfaktorischen Rezeptoren (ORs) zu dem duftgesteuerten Verhalten von *Drosophila melanogaster* zu untersuchen. Ein Kernpunkt beschäftigt sich daher mit der sexuellen intraspezifischen Kommunikation dieser Fliegenart. Während unserer Untersuchungen fanden meine Kollegen und ich eine bisher unbekannte Quelle für die Analyse von Fliegenpheromonen – Fliegenkot (Manuskript II). Unter anderem benutzte ich den Flywalk, ein high-troughput Bio-Assay, welches in unserer Abteilung speziell entwickelt wurde, um zu zeigen, dass *Drosophila melanogaster* den Geruch von Fliegenkot wahrnimmt und diesen auch attraktiv findet. Weitere Untersuchungen ergaben, dass das chemische Profil, welches wir im Kot fanden, dem der Fliegencuticula gleicht. Wir fanden in beiden Quellen die bekannten Fliegenpheromone Methyllaurat, Methylmyristat,

Methylpalmitat und 11-*cis*-Vaccenylacetat (cVA). Außerdem konnten wir zeigen, dass die Attraktion der Fliege zum Kot durch ein Zusammenspiel aller genannten Komponenten zustande kommt. Wenn wir nur eine spezifische Population von olfaktorisch-sensorischen Neuronen ausschalteten, zeigten die Testtiere kein Attraktionsverhalten. Weiterhin präsentierten wir Ergebnisse, die aufdecken, wie dieses sensible System durch parasitäre Bakterien ausgenutzt wird (Manuskript III). Pathogene, welche das Immunsystem der Fliegen aktivieren, sind in der Lage die Menge an produzierten Pheromonen im Wirt zu erhöhen. Auf diese Weise kommt es zu vermehrten Kontakt zwischen den Individuen, was wiederum die Ausbreitungschancen der Bakterien erhöht.

Im zweiten Abschnitt meiner Dissertation beschäftige ich mich mit einem anderen Teil des olfaktorischen Systems von Insekten – der Wahrnehmung und Verarbeitung von Futterdüften. Dazu haben meine Kollegen und ich als erstes untersucht, ob die Larven und adulten Tiere von *Drosophila melanogaster* unterschiedliche Präferenzen haben, wenn ihnen eine bunte Mischung an Früchten präsentiert wird (Manuskript V). In der Tat fanden wir Unterschiede in den beiden Entwicklungsstadien hinsichtlich ihrer Vorlieben. Während die Larven besonders Erdbeeren, Passionsfrucht und Ananas mochten, entschieden sich die adulten Tiere für Johannisbeere, Kiwi und Feige. Interessanterweise, wurden erwachsene Fliegen besonders von den Fruchtdüften angezogen, die die Antennen nur wenig aktivierten, während die Larven Düfte immer dann besonders attraktiv fanden, wenn ihr Dorsalorgan stark gereizt wurde. Offensichtlich, sind die Larven dadurch an ihren Lebensraum in den verrotteten Früchten, wo hohe Duftkonzentrationen herrschen, angepasst. In einer wetieren Studie, die die Verarbeitungen von Duftmischungen untersuchte, versuchten wir den Mechanismus hinter diesem Phänomen zu

verstehen (Manuskript IV). Da Fruchtdüfte im Wesentlichen ein komplexer Mix aus verschiedenen Einzeldüften sind, haben wir einen einfacheren Ansatz gewählt und binäre Mischungen von attraktiven und aversiven Düften analysiert. Dabei fanden wir eine mischungsabhängige Duftantwort der getesteten Tiere, d.h. wenn der positive Duft in der Mischung dominierte, wurde die Mischung als attraktiv wahrgenommen und umgekehrt. In diesem Zusammenhang haben wir auch die Rolle der lokalen Interneurone (LNs) im Antennallobus bezüglich ihrer Beteiligung in der Signalmodulation untersucht. Dabei fanden wir glomerulusspezifische GABAerge Inhibitionsmuster. Nach unserem Wissen sind wir die Ersten, die zeigen konnten, dass manche Glomeruli sowohl pre- als auch postsynaptisch GABAerg inhibiert werden, während andere Glomeruli nur postsynaptische Inhibition erfahren.

Weiterhin habe ich die unterschiedliche Effektivität von neuronalen Effektoren in Abhängigkeit von dem gewählten Bio-Assay gezeigt. Während *reaper* und *diphtheria toxin* in keinem der gewählten Experimente das duftgesteuerte Verhalten der Fliegen aufheben konnten, war die Effizienz von *tetanus toxin* und dem Kaliumkanal *Kir2.1* sehr von dem jeweiligen Expressionslevel, sowie von dem gewählten Bio-Assay abhängig (z.B. Luftstrom/kein Luftstrom). Am Ende war das homozygot exprimierte *tetanus toxin* der einzige getestete Effektor, der den Phänotyp von *orco*-Mutanten vollständig nachahmen konnte. Dieses Ergebnis unterstreicht, wie vorsichtig man bei der Wahl selbst wissenschaftlich voll etablierter genetischer Werkzeuge und der gewählten Verhaltensassays sein muss.

Generell öffnet diese Doktorarbeit mit ihrem besseren Verständnis für die Attraktivität von Düften die Tür zu einer Zukunft, in der z.B. der Kot von Schädlingen genutzt werden

kann, um Lockmittel für eine ökologische Bekämpfung zu entwickeln. Auf diese Weise schützen wir nicht nur die Ernte, sondern auch die Umwelt – Mutter Erde.

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Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctor theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University Jena or to any other university.

_____ _____

Place and date Signature

Curriculum Vitae

Name:	Tom Retzke	
Adress:	Fritz-Büchner-Straße 32 99086 Erfurt	
Date of Birth:	14.07.1988	
Birthplace:	Wolfen, Saxony-Anhalt	
Nationality:	German	
Languages:	English (Negotiable) Russian (Basics) Japanese (Basics)	
Special Abilities:	MS Office, Adobe Illustrator, R	
Career:	2001 – 2007	"Anne-Frank-Gymnasium" Sandersdorf
	2007 – 2010	TU Darmstadt
	2010 – 2013	Friedrich Schiller University Jena
	2014 – 2018	Max Planck Institute for Chemical Ecology
Degrees:	Abitur (Grade: 2.2) Diploma (Grade: 1.1) PhD thesis in preparation	

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Don´t worry, I wrote the names in bold and underlined them, so you can find yourself easier. ;)

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"Don´t clap too hard – it´s a very old building."

John Osborne